

# **Classification and follow-up of Sjögren's syndrome**

## **Usefulness of objective parameters in clinical practice**

Een wetenschappelijke proeve  
op het gebied van de Medische Wetenschappen

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# CONTENTS

<b>Chapter 1</b>	General introduction	5
	1.1 The evolving concept of Sjögren's syndrome diagnosis	
	1.2 Pathogenesis and pathophysiology of Sjögren's syndrome	
	1.3 Can disease activity be assessed in Sjögren's syndrome ?	
	1.4 Treatment: localized and systemic approaches	
	1.5 Aims and outline of the thesis	
<b>Chapter 2</b>	Tear fluid measurement of anti-SS-A and anti-SS-B antibody in anti-SS-A and anti-SS-B seronegative Sjögren's syndrome patients (submitted)	27
<b>Chapter 3</b>	Anti-alpha-fodrin antibodies do not add much to the diagnosis of Sjögren's syndrome Arthritis Res Ther 2004; 6:R33-R38	35
<b>Chapter 4</b>	Estrogen-beta receptor expression in skin, salivary glands and salivary gland infiltrating lymphocytes in Sjögren's syndrome patients (submitted)	47
<b>Chapter 5</b>	The synergistic value of focus score and IgA% score of sublabial salivary gland biopsy for the accuracy of the diagnosis of Sjögren's syndrome: a 10-year comparison Rheumatology 2002 41: 819-823	59
<b>Chapter 6</b>	Reversibility of histological and immunohistological abnormalities in sublabial salivary gland biopsies following treatment with corticosteroids in Sjögren's syndrome Ann Rheum Dis 2001 60: 511-513	69
<b>Chapter 7</b>	Etanercept in the treatment of primary Sjögren's syndrome; a pilot study J Rheumatol 2004 31:96-101	77
<b>Chapter 8</b>	Summary	91
<b>Chapter 9</b>	Samenvatting	99
<b>Appendix</b>	Sjögren's syndrome criteria [letter] Ann Rheum Dis 2002;61:948-949	107
	Dankwoord	109
	Curriculum vitae	111
	List of publications	112



## **CHAPTER 1**

# **General introduction**

- 1.1 The evolving concept of Sjögren's syndrome diagnosis**
- 1.2 Pathogenesis and pathophysiology of Sjögren's syndrome**
- 1.3 Can disease activity be assessed in Sjögren's syndrome ?**
- 1.4 Treatment: localized and systemic approaches**
- 1.5 Aims and outline of the thesis**

## 1.1 - The evolving concept of Sjögren's syndrome diagnosis

Sjögren's syndrome (SS) is considered a chronic autoimmune exocrinopathy, particularly involving salivary and lacrimal glands. As a result SS patients experience so called sicca symptoms such as dry eyes and dry mouth. In addition, exocrine glands that are located in the upper airways, the vagina and possibly the skin may also be affected. SS is thought to affect approximately 0.5-1.0% of the population in the US and Europe, and has been subject of extensive scientific research. One of the continuing dilemmas in both research and daily clinical practice however is how to define SS. As a consequence, data obtained can often not be easily compared. Importantly, whereas some physicians diagnose SS by using classification criteria sets, others prefer to establish a diagnosis based on the clinical symptoms and signs that constitute the syndrome. This paper deals with the evolving concept of SS, starting from the early descriptions of the syndrome by Henrik Sjögren and others, describing the various classification criteria and consecutive modifications that have been applied to them, and finishing with possible future developments and their implications for both research and daily clinical practice.

### **The early years:**

#### **descriptions of clinical and histopathological features of the syndrome**

In medicine a *syndrome* is defined as a group of symptoms that collectively indicate or characterize a disease or abnormal condition. Both clinical and histopathological features of the medical condition nowadays known as *Sjögren's syndrome* have been described over a century ago. In 1888 the London physician Halden<sup>1</sup> described the concurrent presence of dry eyes and dry mouth in a 65-year-old woman also suffering from loss of taste and smell. When she was treated with tincture of jaborandi (pilocarpine), her mouth became much more moist. Also in 1888, the polish surgeon Mickulicz<sup>2</sup> noticed a combined and similar salivary and lacrimal gland pathology. He demonstrated the presence of a round cell (lymphocytic) infiltration into both the enlarged parotid and lacrimal glands of a 42-year-old male farmer. Because Mickulicz described histopathological abnormalities without an explicit association with clinical features, a number of distinct diseases leading to salivary gland enlargement and lymphocytic infiltration was then labelled Mickulicz disease. Sixty-five years later Morgan and colleagues concluded that the patient presented by Mickulicz had in fact the same histopathological features as then seen in SS patients<sup>3</sup>. It also took some decades since the first observations by Halden and Mickulicz, before others not only combined these symptoms and signs to a syndrome, but also explicitly hypothesised the syndrome to be caused by an underlying systemic disease. In an interview Henrik Sjögren, whose name is currently linked to the syndrome, stated that his major contribution has been the recognition of the sicca syndrome as a systemic disease<sup>4</sup>. By describing keratoconjunctivitis sicca in patients with chronic polyarthritis the Dutchman Mulock Houwer<sup>5</sup>, and the Swedish ophthalmologist Henrik Sjögren<sup>6</sup> noted characteristics

for an underlying systemic disease responsible for the syndrome, whilst the French physician Gougerot<sup>7</sup> presumed a generalized exocrinopathy. Since exocrinopathy is still considered as a major characteristic of SS, it might have been named after the French physician as well. Previously the term Gougerot-Sjögren syndrome has indeed been used in literature quite often. However, both Sjögren and Gougerot failed to further specify whether the suggested underlying systemic autoimmune disease was a specific and distinct disease entity or whether this could be any form of previously known systemic disease. In other words: does a distinct pathophysiological mechanism lead to SS, or can several different pathophysiological mechanisms all lead to SS, which could then be considered as a final common pathway of multiple systemic diseases? By leaving this issue open the question how to define SS became an ongoing dilemma. In 1965 Bloch and Buchanan postulated the definition that SS consists of the triad of keratoconjunctivitis sicca (KCS), xerostomia and rheumatoid arthritis (RA) or any other connective tissue disease<sup>8</sup>. This clinical definition has also considerably influenced the continuing debate on the definition of SS. Since two of the three components were sufficient for the diagnosis, in clinical practice a rather heterogeneous group of patients was labelled as having SS.

### **A heterogeneous SS population: the need for classification criteria**

In the years following the work of Sjögren and Gougerot a wide range of symptoms has been added to the clinical concept of SS. The suggested underlying systemic disease remained to be further specified. Although objective but non-specific tests, such as Schirmer's test, tear drop break-up time or rose bengal cornea staining, were applied to diagnose KCS, histopathological examination was not frequently performed yet. In the absence of objective evidence of a specific systemic disease (e.g. serological or histological markers) sicca syndrome appeared to be equivalent to Sjögren's syndrome. Indeed, according to the Bloch definition of SS, for the systemic disease component any connective tissue disease would do as long as the patient had KCS and/or xerostomia. Whether such a designation of Sjögren's syndrome accurately identified a specific group of patients with a common pathogenesis or prognosis is questionable, as was recognised more recently by Fox<sup>9</sup>. Meanwhile continuing research had provided accumulating serological and histological data suggestive of different underlying pathophysiological mechanisms within the SS population. Whereas a dense glandular lymphocytic infiltrate, such as described by Mickulicz, is present in the majority of SS patients and can be quantified in a lymphocytic focus score (LFS), in RA patients supposed to have SS as well, such a histopathological finding is often lacking. Thus, in clinically virtually undistinguishable sicca syndrome patients distinct histopathological features are found. Vice versa not all lymphocytic infiltrates necessarily reflect the same medical disease. E.g. a LFS > 1.0 can be found in SS, but also in other medical conditions such as RA, SLE, scleroderma, graft-versus-host disease, AIDS, myasthenia gravis, primary biliary cirrhosis and, in fact, in 5-10% of the normal population<sup>10</sup>

Although immunohistological staining for the composition of these infiltrates (e.g. determining the percentage of IgA or IgM producing plasma cells) appears to offer better disease specificity than assessing the LFS, this procedure has not been widely applied yet<sup>11-13</sup>. Thus, whilst the LFS provided an objective tool to distinguish different pathophysiological conditions all leading to the sicca syndrome, its role in SS definition was much debated because of the relative lack of fine disease specificity.

Similar controversies occurred following the detection of anti-SS-A and anti-SS-B autoantibodies in Sjögren's syndrome in 1960<sup>14</sup>. Serological assessment provided important objective markers of the systemic component of the disease. The observed distinct serological profiles enabled subgroup definition and, similar to the LFS findings, provided indications for the presence of distinct pathophysiological mechanisms that lead to the sicca syndrome. However the disease sensitivity and specificity of the classic anti-SS-A and anti-SS-B antibodies, as well as those of suggested alternative antibodies, such as e.g. anti-muscarinic receptor or anti-fodrin antibodies<sup>15-18</sup>, has been limited. Nevertheless, it was obvious that the systemic disease component differed amongst the patients diagnosed with SS using the clinical Bloch definition. Since Henrik Sjögren did not speculate on the nature of a specific underlying systemic disease and the Bloch definition did not conflict with findings of distinct underlying systemic diseases, why should the term Sjögren's syndrome be preserved for a subgroup of patients? On the other hand, to get on with research, having recognised distinct pathophysiological conditions, there was a need for consensus on how to classify SS. As a result, in the early 80s several classification criteria sets for SS were introduced. These sets included both clinical signs and symptoms of dry eyes and mouth and pathophysiological signs such as salivary gland histology and serological evidence for an underlying systemic disease. Note that these classification criteria are quite compatible with the Bloch concept and not based on the concept of SS as a specific pathophysiological disease entity on its own. Inevitably, the SS population continues to be heterogeneous, however due to serological and histological specifications of the systemic component to a lesser extent than before.

### **The introduction, modifications and merging of SS classification criteria**

In 1986 a number of classification criteria for SS was proposed at the first International Sjögren's Syndrome Symposium that, given the history mentioned above, was mainly focused on diagnostic issues. Regarding the use of classification criteria no consensus was reached, and as a result all of the presented classification criteria were used in the years following this symposium. This meant that over the years at least five main classification criteria sets were used in international research: two US sets (Fox' San Diego and Daniels-Talal's San Francisco criteria)<sup>19,20</sup>, two European sets (Copenhagen and Greek criteria)<sup>21,22</sup> and a Japanese set<sup>23</sup>. Fortunately continuing discussions and accumulating study data have led to a more uniform diagnostic approach nowadays. The two European



classification criteria sets soon merged to one so called European Study Group classification criteria set<sup>24</sup>. A decade later, at the VIIIth international Symposium on Sjögren's syndrome, a combined US/European classification criteria set was introduced<sup>25</sup>. Unfortunately the Japanese classification criteria have not yet been included in this new international classification criteria set. In order to come to a single, worldwide applied, classification criteria set for Sjögren's syndrome, a final merging of classification criteria sets is scheduled for the next symposium. In the current US/European classification criteria presence of either anti-SS-A/anti-SS-B antibodies or a focus score >1 is obligatory for the classification Sjögren's syndrome. Thus, whilst the usefulness of both of these parameters was much debated in 1986, they have nowadays evolved to obligatory items.

### **Primary and secondary SS: a confusing terminology**

In the absence of serological or histological evidence for SS, some patients previously diagnosed as having SS were now considered sicca syndrome patients. Considering Bloch's clinical definition of SS, a patient with RA, xerostomia and KCS could nevertheless still have SS. By introducing the terms primary and secondary SS this problem was bypassed. However, the agreed upon classification criteria for secondary SS were less stringent than those for primary SS and did not require presence of either anti-SS-A or anti-SS-B or positive focus score<sup>26</sup>. As a consequence, the term secondary SS still covered a heterogeneous population of patients with either an overlap syndrome of the now as primary SS defined condition and an other connective tissue disease or sicca syndrome secondary to e.g. RA. This is reflected by both pathophysiological and clinical differences. Primary SS is often associated with the HLA-DR3 phenotype. In contrast, secondary SS patients with RA are often linked to HLA-DR4<sup>27-29</sup>. Whilst presence of either anti-SS-A or anti-SS-B antibody is observed in approximately 80% of primary SS patients, secondary SS patients are frequently ANA positive, but not anti-SS-A or anti-SS-B positive<sup>30</sup>. In addition, recently an association between anti-SS-A/SS-B antibody production and HLA-DR3 was reconfirmed<sup>31,32</sup>. Furthermore, as mentioned previously, a positive salivary gland biopsy (focus score >1) is less often observed in secondary SS as compared to primary SS. Moreover, determining the percentage of IgA containing cells in salivary gland infiltrates has been shown to enable discrimination between rheumatoid arthritis patients with sicca syndrome and true Sjögren's syndrome patients<sup>11</sup>. In the course of the disease primary SS patients may typically develop central or peripheral nervous system involvement, hypergammaglobulinemia, purpura, or a malignant lymphoma. In contrast these signs are often absent in secondary SS patients whose sicca symptoms may be relieved by treatment of the underlying connective tissue disease (e.g. RA)<sup>33</sup>. However, for primary Sjögren's syndrome there is no causal treatment strategy available yet. Thus, using the term "overlap syndrome" in case a primary SS patient also has an other connective tissue disease appears preferable, since from a pathophysiological point of view Sjögren's syndrome is not causally related to any

other connective tissue disease and is therefore not “secondary”. The terms “SS”, “SS overlap syndrome” (e.g. SS/SLE) and “non-SS sicca syndrome” provide a more unambiguous nomenclature and shall therefore be used throughout this thesis.

### **Early diagnosis as possible future development: implications for the classification criteria ?**

More than a century after the first pathological descriptions by Mickulicz a trend from a long-lasting emphasis on clinical signs back towards pathophysiological signs can be noted in the evolving concept of Sjögren's syndrome. Either serum presence of anti-SS-A and/or anti-SS-B antibodies. or positive sublabial or parotid salivary gland biopsy is mandatory in the current classification criteria and outlines a particular patient population. This trend may eventually lead to diagnosing Sjögren's syndrome at an earlier stage, possibly enabling reversibility of glandular pathology. Presence of anti-SS-A or anti-SS-B antibodies has also been observed in saliva and tears of SS patients<sup>34-37</sup>. Apart from antibody profiles, characteristic early changes in composition of saliva or tears are also subject of continuing research activities<sup>38</sup>. In animal models early histopathological changes are increasingly documented<sup>39,40</sup>. When knowledge about the natural (slowly progressive) course of the disease accumulates and more tools for early diagnosis become available, the classical sicca symptoms may eventually prove to be rather non specific end-stage disease manifestations. Following new insights the history-based model of Sjögren's syndrome needs to be reconsidered. Implementation of these developments in daily clinical practice will be challenging. The end-stage disease sicca symptoms have been the “trigger” for doctors to check whether the patient has Sjögren's syndrome. So far however, following a diagnosis no lasting curative treatment can be offered to the patient. In order to diagnose the syndrome before irreversible glandular damage occurs, a screening test for Sjögren's syndrome will have to be considered in a “pre-sicca” stadium in which the patient may only present with non-specific symptoms. This demands a quick, cheap, preferably non-invasive specific and sensitive screening test. For the moment, the need for early diagnosis before irreversible damage has taken place, may not be widely felt before disease-modifying treatments are available.

## **1.2 - Pathogenesis and pathophysiology of Sjögren's syndrome**

Sjögren's syndrome (SS) is generally considered an autoimmune exocrinopathy. Despite extensive research the exact pathogenesis of this exocrinopathy remains to be elucidated. Various patho-physiological disease models for Sjögren's syndrome have been suggested and explored. Apart from an underlying genetic predisposition, endocrine, neurological, and environmental factors, as well as infectious agents have been put forward as potentially triggering and maintaining primary Sjögren syndrome.

### **Immunogenetic factors in the pathogenesis of Sjögren's syndrome**

A scale of genes encoding for different components of the immune system can potentially account for the genetic predisposition that appears to be present in SS<sup>27</sup>. These include histocompatibility genes, T-Cell receptor (TCR) genes, immunoglobulin genes, antigen-processing and peptide transport genes and complement genes<sup>41</sup>. Histocompatibility genes are involved in the formation of the T cell repertoire from the thymus, whilst TCR genes have a role in determining the number and specificities of autoreactive T cells. Immunoglobulin genes are responsible for regulation of specificities and idiotypes of natural autoantibodies. Inefficiently coding antigen-processing and peptide transport genes can impair the development of anergy whilst defects in complement genes may lead to complement deficiency. In other words, polymorphisms in any of these genes may code for gene products that dysregulate the normal immune function, and thereby predispose for autoimmune attack.

Although meanwhile additional genes and their encoded molecules have been explored (e.g. genes encoding for apoptosis, or cytokines) none have been as much explored in autoimmunity research as the histocompatibility or HLA genes. A variety of HLA-DR and later HLA-DQ alleles have been associated with most of the autoimmune diseases, including SS. HLA-DR3 and HLA-Dr2 associations have been found in western European SS patients and patients with an overlap SS/SLE or SS/systemic sclerosis<sup>42</sup>. Interestingly patients with RA and secondary sicca syndrome have been associated with HLA-DR4 but not HLA-DR3 or HLA-DR2<sup>43</sup>. It has been noted however that HLA-DR and HLA-DQ associations differ in distinct ethnic groups. In Greek patients and Israeli Jews e.g. SS is rather associated with the DR5 allele. Japanese SS patients show other associations<sup>44,45</sup>. This raises the question whether SS patients are a genetically heterogeneous group or whether SS patients differ as a result from various diagnostic criteria sets applied in these countries. HLA-DR and especially DQ alleles also seem to be involved in autoantibody formation (e.g., against SS antigen-A/Ro and SS antigen-B/La)<sup>31,32</sup>. Synthesis of autoantibodies against B-cell epitope analog of SSB/La in Caucasian patients with primary SS seemed to be dependent on the presence of a permissive HLA-DQ heterodimer, most prominently represented by HLA-DQA1\*0501/DQB1\*0201. As a result of this finding, a model of HLA-restricted presentation of SSB/La peptide determinants has been suggested<sup>46</sup>.

### **Endocrinological factors in the pathogenesis of Sjögren's syndrome**

Since Sjögren's syndrome is a disorder predominantly affecting women a role for sex steroids in the pathogenesis has been assumed. The fact that a number of immunoregulatory features of estrogens have been described provides additional support for this hypothesis. However, previous studies did not elucidate any endocrine mechanisms directly involved in the pathogenesis of SS or SS/SLE overlap syndrome. A large proportion of androgens and estrogens in men and women are synthesized locally in peripheral target tissues from the inactive adrenal precursors

dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S). In postmenopausal women, 100% of active sex steroids are synthesized in peripheral target tissues from inactive steroid precursors. However, in a pilot clinical trial DHEA supplement treatment showed no evidence of efficacy in primary SS patients<sup>47</sup>. Estrogen activity has been linked to cutaneous expression of the classic SS antibodies anti-SS-A and anti-SS-B. Estradiol has been reported to enhance binding to cultured human keratinocytes of antibodies specific for SS-A and SS-B<sup>48-50</sup>. It must be noted that all studies were performed *in vitro* in an experimental setting using cultured cells.

Meanwhile, several authors have reported on different localizations and functioning of two distinct estrogen receptor (ER) types, named ER- $\alpha$  and ER- $\beta$ , in human tissues. These studies indicate that ER $\beta$  is the predominant ER in the skin, salivary glands and oral epithelium<sup>51-53</sup>. This is of particular interest if confirmed in SS patients specifically since SS typically becomes manifest in salivary glands and oral epithelium. A possible role for ER- $\beta$  in triggering or maintaining SS could then be hypothesized.

Instead of focussing on estrogen activity, androgen-deficiency might offer an alternative explanation for the sex differences in SS prevalence, as suggested in recent studies<sup>54,55</sup>. Interesting observations come from case studies of patients suffering from Klinefelter's syndrome (KS). Three patients with KS and SS showed a clinical remission of SS and a decrease in ANA and RF titers as well as ESR following treatment with the androgen T undecanoate<sup>56</sup>. In KS patients testosterone replacement treatment has also been found to decrease serum levels of IgA, IgG, IgM, IL-2 and IL-4<sup>57</sup>. This suggests that the lack of testosterone in patients with KS enhances cellular and humoral immunity and that androgen replacement therapy may suppress this. Next to these observations pregnancy, the use of oral contraceptives, and postmenopausal hormone replacement therapy all affect the course of autoimmune diseases differently. Therefore the role of sex steroids in SS remains an interesting though complex matter that needs further research, especially following the recent discovery of a second estrogen receptor ER $\beta$  and the recognition of tissue specific intracellular steroidogenic enzyme activity.

### **Neurological factors in the pathogenesis of Sjögren's syndrome**

Sicca symptoms in SS have commonly been attributed to lymphocytic infiltration and destruction of the lacrimal and salivary glands<sup>58</sup>. However, the severity of sicca symptoms does not always correlate with the degree of glandular destruction seen in labial biopsy tissue<sup>59</sup>. The residual glandular elements in the salivary gland appear dysfunctional even though they apparently maintain their neural innervation<sup>60</sup> and despite upregulation of their muscarinic receptors<sup>61</sup>. It is hypothesized that this suboptimal function of the residual acinar and ductal cells, leading to sicca symptoms, may result from local action of e.g. cytokines, autoantibodies and metalloproteinases or from parasympathetic neural dysfunction<sup>62</sup>. Features suggestive of dysfunction of the autonomic nervous system,

including Adie's tonic pupil<sup>63</sup>, fluctuating blood pressure and orthostatic hypotension<sup>64</sup>, anhidrosis<sup>65</sup>, and constipation<sup>66</sup>, have been reported to occur in SS. A prospective study of 32 SS patients showed presence of autonomic neuropathy in the majority of patients<sup>67</sup>. Bladder symptoms have also been reported, including frequency of micturition, nocturia, urgency, and incomplete bladder emptying, frequently in association with interstitial cystitis<sup>68</sup>. Muscarinic receptors of the M3 subtype mediate the stimulatory effects of acetylcholine on the lacrimal and salivary glands, as well as the contractile effects of acetylcholine on the bladder and intestinal smooth muscle and on nitric oxide production in blood vessels. Salivary IgA autoantibodies against M3 muscarinic acetylcholine receptors have been proposed as a marker to differentiate Sjögren syndrome from non-Sjögren syndrome xerostomia<sup>69</sup>, whereas serum IgG autoantibodies against the M3 receptor have recently been shown to reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells<sup>70</sup>. Therefore, muscarinic receptors have been identified as a rational target of drug therapy by specific agonists.

### **Infectious factors in the pathogenesis of Sjögren's syndrome**

Several infectious agents have been suggested to trigger SS. Bacterial and mycotic agents present in the oral cavity or in salivary cultures have been identified as related to SS<sup>71-73</sup>. However whether these agents were the cause or rather the result of SS can be questioned. Viral agents, in contrast to other infectious agents have drawn much more attention in SS research. Hepatitis C virus (HCV), herpes viruses (e.g. Epstein-Barr virus, cytomegalovirus, human herpes virus-6), and retroviruses (AIDS HTLV-1) have all been linked to SS<sup>74,75</sup>. Herpes viruses and retroviruses have sialotropic and lymphotropic properties. Their localization to salivary glands could lead to primary pathology and lymphotropism could lead to dysregulation of the immune system. However, not any of the proposed viral triggers was detected constitutively in patients with Sjögren syndrome or was specific for Sjögren syndrome.

### **Pathophysiological aspects of Sjögren's syndrome**

B cell hyperreactivity and lymphoplasmacytic infiltrations of exocrine glands are the major autoimmune characteristics of SS<sup>76</sup>. Glandular destruction in SS has been shown to be mediated by primed CD4+ T helper and inducer lymphocytes<sup>77</sup> which may also explain high B cell activity. However, salivary and lacrimal glands from SS patients have an impaired secretory function, even during the initial phase of inflammation and onset of lymphocytic destruction. Next to structural loss of glandular elements and parasympathetic neural dysfunction, the release of proinflammatory cytokines (such as TNF-alpha) by both lymphocytes and glandular cells, induction of matrix metalloproteinases<sup>78</sup>, as well as an impaired release of and response to neurotransmitters may be involved in the loss of secretory function of the residual glandular cells in SS<sup>79</sup>.

Systemic autoimmune diseases such as Sjögren syndrome, systemic lupus erythematosus, and rheumatoid arthritis appear to have characteristic patterns of B-lymphocyte distribution<sup>80</sup>. B cell activity in the salivary glands enables quantitative immunohistological evaluation of submandibular salivary gland biopsy specimens. Measurement of the number and distribution of immunoglobulin isotype producing plasmacells has been shown to be superior to lymphocytic focus score assessment in the diagnosis of SS<sup>11-13</sup>. In SS patients a significant decrease in the frequency of peripheral memory CD27+ B cells<sup>81</sup> has been found. Whether the reduction of circulating CD27+ memory B cells in SS results from disturbed B-cell differentiation<sup>82</sup>, preferential trafficking or homing of memory B cells into target tissues<sup>83</sup>, or alternative B-cell activation pathways via toll-like receptors<sup>84</sup> is not known yet. Recently promising results of anti-B-cell treatment strategies in SS have been reported<sup>85,86</sup>.

Autoantibodies are frequently found in SS and directed to a wide range of antigens. The significance of individual autoantibodies and their potential role in triggering or maintaining SS has been much studied as well as debated. So far, although also found in SLE, antibodies directed to the SS-A (Ro) and SS-B (La) antigen are considered as the most disease sensitive (60–75% and 30–50%, respectively) and specific serological markers of SS. As a consequence anti-SS-A and anti-SS-B play an important role in current classification criteria for SS. Although antibodies targeting a variety of candidate autoantigens (more recently alpha-fodrin and muscarinic M3 receptor) have been suggested as alternative disease markers, none of them has proven to be superior to anti-SS-A and anti-SS-B<sup>15-18</sup>. Nevertheless, in approximately 20% of SS patients no anti-SS-A and anti-SS-B can be detected at the time of diagnosis. In conclusion, SS-associated autoantibodies although significantly contributing, do not provide the golden standard for the objective diagnosis of SS.

### **Natural course of Sjögren's syndrome**

Unlike other rheumatic diseases, such as rheumatoid arthritis and SLE, the clinical course of SS tends to be stable and is characterised by unchanged or slowly deteriorating exocrine gland function over time. Exacerbations or “flares” are rather infrequently seen or reported in the course of SS. In a recent study only 3-7% of a cohort of SS patients (as compared to 62% in SLE) reported having had one or more flares per year<sup>87</sup>. Fatigue and arthralgia were the main constituents of reported flares in this population. Few longitudinal studies in SS have been published. Kruize<sup>88</sup> performed a 10-12 year follow-up study and concluded that SS is characterized by a stable and rather mild course of glandular and extraglandular manifestations. During follow-up 3 of 30 SS patients developed and died of malignant lymphoma. Similar reports come from a study in the North-East of England Davidson<sup>89</sup>. Stable clinical features were observed in 100 SS patients following 10 years of follow-up. Three of these patients, all anti-SS-A and anti-SS-B positive, developed malignant lymphoma. For anti-SS-A and anti-SS-B positive

patients in this follow-up cohort the relative risk of developing malignant lymphoma was 49. Gannot<sup>90</sup> also noted a stable clinical course in a 5-10 year follow-up study of 80 SS patients. Despite the observed clinically mild course, these longitudinal studies all confirmed that SS patients are at significantly increased risk to develop malignant lymphoma (see below).

### **Lymphomagenesis**

Sjögren syndrome patients are at increased risk to develop B-cell lymphomas, which occur 16 to 44 times more frequently in patients with SS than in normal healthy subjects. Most commonly the lymphoma is of the mucosa-associated lymphoid tissue (MALT) type<sup>91-93</sup>. These MALT lymphomas are thought to originate in the organs targeted by Sjögren syndrome and spread to other mucosal sites over time<sup>93-95</sup>. Based on recent long-term outcome studies in primary Sjögren syndrome patients, the presence of palpable purpura, low C4 levels and a low CD4+/CD8+ T lymphocyte ratio have been proposed as predictive factors distinguishing patients at high risk for developing lymphoma from patients with an uncomplicated disease course<sup>92,96</sup>.

## **1.3 - Can disease activity be assessed in Sjögren's syndrome ?**

### **Reversibility in Sjögren's syndrome**

Reversibility is an essential component of the currently proposed disease activity model<sup>97</sup>. However, in SS objectively documented clinical or histological reversibility is restricted to a few case reports. Because therapeutic interventions are often based on an anti-inflammatory approach this might reflect lack of an active inflammatory process. Lack of inflammation might result from SS diagnosis in a relatively late stage of the disease or from an active underlying disease process that is not accompanied by inflammation. The latter hypothesis might be supported by the reported improvement of Schirmer tests following pilocarpin treatment. However, the reproducibility of Schirmer-tests and their correlation to clinical symptoms has been questioned<sup>98</sup>.

Systemic symptoms such as fatigue and arthralgia play a prominent role in SS patients and are a target of therapy. However, reversibility of these features can not be objectively evaluated and has to be assessed by e.g. questionnaires and patients reports. In concordance with the described non-fluctuating slowly progressive course of SS, significant changes in laboratory parameters such as antibody levels, ESR, hypergammaglobulinemia, are not frequently observed.

To date, case reports describing reversibility of histological changes in biopsy specimens following corticosteroid or anti-B-cell treatment provide the sole objectively documented examples of reversibility in SS. In two case reports normalisation of histopathological changes following high dose corticosteroid treatment is observed<sup>99,100</sup>. A similar normalisation was noticed in a female SS



patient suffering from interstitial nephritis. After high dose corticosteroid treatment renal biopsy showed a markedly improved picture. Changes in salivary gland immunohistology and function following anti-B cell monotherapy have recently been shown in a patient with SS and MALT lymphoma<sup>101</sup>. Since reports on reversibility in SS are restricted to individual case reports it remains to be elucidated to what extent clinical and histological symptoms and signs are reversible in SS.

### **Disease activity**

Although no effective systemic treatment is available to date it must be noted that in clinical trials treatment efficacy is mainly clinically evaluated using a wide range of non-uniform objective and subjective parameters. A need for an uniform objective disease activity parameter is widely felt. However, since both the definition of Sjögren's syndrome and knowledge of its pathophysiology are evolving as described before, establishing and validating a widely agreed upon disease activity parameter is easier said than done. In March 2000 collaborative research into outcome measures in Sjögren's syndrome was initialized<sup>102</sup>. Several workshops and consensus meetings have led to proposals of two disease monitoring tools for Sjögren's syndrome, the BILAG-SS and the ECLAM-SS (not published yet). The BILAG-SS has already been applied in an analysis of anti-SS-A and anti-SS-B antibody levels and their relation to disease activity<sup>103</sup>. Both the BILAG-SS and ECLAM-SS are modified versions of disease activity tools for monitoring SLE (ECLAM stands for European Consensus Lupus Activity Measurement whilst BILAG stands for British Isles Lupus Assessment Group). Recently a prospective study was presented showing that the total scores of both BILAG-SS and ECLAM-SS correlate well with each other<sup>104</sup>. Since a disease activity monitoring tool specifically designed for SS is still lacking this appears a promising approach. Disease monitoring tools that have previously been developed for RA and SLE have led to significant advances in research and follow-up studies of these conditions. However, in validating disease activity tools for SS several disease-specific difficulties will be encountered and will have to be solved. As mentioned previously, in contrast to e.g. In RA or SLE, exacerbations or "flares" are rather infrequently seen or reported in the course of SS and not associated with elevated ESR or CRP levels<sup>87</sup>. Longitudinal studies confirm the stable clinical course of SS. The apparent non-fluctuating slowly progressive course and the lack of measurable exacerbations makes defining and monitoring disease activity in SS a difficult issue. In developing a disease activity tool for SS these stable disease course features were recognised. Therefore the idea of disease activity solely based upon inflammation was left behind. This is in concordance with the fact that disease activity as perceived by the patient is often not accompanied by increased inflammatory parameters. Disease activity has now been defined as those disease characteristics that are potentially reversible, regardless of the pathogenic mechanism, as opposed to damage which by definition is irreversible<sup>105</sup>. Apart from the fact that little is known about reversibility in Sjögren's syndrome, this approach



has led to a prominent role for arthralgia and fatigue as (indirect) markers of systemic disease activity. However, these symptoms are inevitably subjective of nature and unlike e.g. arthritis do not lead to end-organ damage. Defining a disease severity (or damage) index to start with may be more suitable given these considerations. However, so far only a single study of end-organ damage has been performed in SS patients<sup>106</sup>. A proper definition of end-organ damage in SS may be helpful in the further development of a disease activity tool.

#### **1.4 - Treatment: localized and systemic approaches**

Thus far, no effective systemic treatment is available for SS. Disease-modifying anti-rheumatic drugs (DMARDs) which are successfully applied in the treatment of several other rheumatic diseases did not turn out to be a successful overall approach in SS<sup>107-109</sup>. In some cases glucocorticoids are reported to offer some subjective improvement such as a decrease of fatigue complaints, but the efficacy of corticosteroid treatment in SS has not been objectively documented in placebo-controlled studies<sup>110</sup>. Patients with Sjögren's syndrome also encounter greater problems with corticosteroids, including acceleration of periodontal disease and oral candidosis. The use of corticosteroids in SS is therefore restricted to treat life-threatening complications, such as severe vasculitis or interstitial nephritis. Hydroxychloroquine can be useful in decreasing arthralgia, myalgia, and lymphadenopathy<sup>111</sup>. Kruize and colleagues noticed that following hydroxychloroquine treatment ESR decreased, but tear flow volumes did not increase<sup>112</sup>.

In the absence of effective systemic treatment SS is mainly treated symptomatically and locally using eye droplets and artificial saliva. Furthermore M3 muscarinic receptor agonists such as pilocarpine and cevimeline may enhance autologous production of both saliva and tears in a subgroup of SS patients<sup>113,114</sup>. Recently new biological agents that are already applied in the management of various other autoimmune diseases such as rheumatoid arthritis and Crohn's disease, have been evaluated in SS. Examples include tumour necrosis factor (TNF)-alpha inhibitors<sup>115-117</sup> and anti-B cell agents. Promising results have been reported concerning the efficacy of anti-CD20 treatment in SS<sup>85,86</sup>. However the ideal treatment modality for SS has yet to be developed.

## 1.5 - Aims and outline of the thesis

In the recently presented combined US/European classification criteria for SS more weight is put on presence of disease characteristic serological and histological signs. These include the serum presence of anti-SS-A- and anti-SS-B-antibody (anti-SS-A, anti-SS-B), as well as the extent of lymphocyte infiltrates in salivary gland biopsy specimens, expressed in a so-called lymphocytic focus-score (LFS). Since in approximately 20% of SS patients no serum presence of anti-SS-A and anti-SS-B can be detected at the time of diagnosis, the additional value of repeated serological examination in SS patients was evaluated after disease had progressed for at least 5 years. Because exocrine gland products can also show (enriched) anti-SS-A and anti-SS-B presence, the tear fluid of seronegative SS patients was analysed for presence of anti-SS-A or anti-SS-B. The potential additional diagnostic value of anti-alpha-fodrin antibodies as alternative serological marker for SS was evaluated.

In salivary gland biopsy specimens the LFS was compared to the percentage IgA containing plasmacells, after both parameters had been assessed for 10 years in our hospital. Reversibility of these (immuno)histological characteristics following anti-inflammatory treatment was evaluated. For this purpose, repeated salivary gland biopsy was performed both before and after anti-TNF-alpha treatment in an open label pilot study in SS patients. Whether a skin biopsy could serve as an alternative for salivary gland biopsy by revealing similar exocrinopathy characteristics was also explored. Thus, in concordance with the increased emphasis on serological and histological signs in recent classification criteria, this thesis is aimed at optimised use of objective signs in the diagnosis of Sjögren's syndrome.

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## **CHAPTER 2**

# **Tear fluid measurement of anti-SS-A and anti-SS-B antibody in anti-SS-A and anti-SS-B seronegative Sjögren's syndrome patients**

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(submitted)

## **ABSTRACT**

### ***Objective:***

Approximately 20 percent of Sjögren's syndrome (SS) patients lack serum presence of anti-SS-A and anti-SS-B antibodies (anti-SS-A, anti-SS-B), which is an emphasized item in the US/European Consensus Group (EUCG) classification criteria for SS. However, anti-SS-A and anti-SS-B can also be found in saliva and tear fluid, as shown previously in SS patients with a positive serological profile. The aims of this study were to evaluate presence of anti-SS-A and anti-SS-B in the tear fluid of SS patients without serum presence of these antibodies, and to re-assess their serological profile after at least 5 years of disease duration.

### ***Methods:***

Tear fluid presence of anti-SS-A and anti-SS-B was assessed by ELISA in 13 seronegative SS patients. Tear samples of three seropositive SS patients served as control. All SS patients fulfilled the EUCG classification criteria. New blood samples were taken from 17 previously seronegative SS patients, all with a disease duration of at least five years. Presence of anti-SS-A or anti-SS-B in these serum samples was measured using counter-immuno-electrophoresis and ELISA.

### ***Results:***

Tear fluid presence of anti-SS-A was found in 2 of 13 seronegative SS patients. Both anti-SS-A and anti-SS-B were found in the tear fluid of all 3 seropositive SS patients. No sero-conversion was observed in seronegative SS patients.

### ***Conclusion:***

In seronegative SS patients tear fluid analysis, in contrast to repeated serological analysis, might reveal anti-SS-A presence, thereby facilitating the diagnosis of Sjögren's syndrome.

## INTRODUCTION

In the current US/European Consensus Group (EUCG) classification criteria for Sjögren's syndrome (SS) objective items, such as serum presence of anti-SS-A and anti-SS-B antibodies (anti-SS-A, anti-SS-B), are emphasized<sup>1</sup>.

In the sera of approximately 20% of SS patients no anti-SS-A or anti-SS-B can be detected at the time of diagnosis. However, in these 'seronegative' patients anti-SS-A or anti-SS-B may nevertheless be present in other body compartments, e.g. within the exocrine glands. Various indications for local autoantibody synthesis in salivary glands of SS patients have been reported previously<sup>2-4</sup>. Therefore it has been recognized that in SS patients anti-SS-A or anti-SS-B might also be detected in the products of the major target organs, i.e. in saliva or tears. However, to our knowledge anti-SS-A or anti-SS-B presence in the tear fluid of SS patients has only been reported once<sup>5</sup>. Interestingly, anti-SS-A and anti-SS-B presence was also demonstrated in the tear fluid of 1 of 12 and 4 of 14 seronegative SS patients respectively. In contrast to this single report on tear fluid, anti-SS-A or anti-SS-B presence in the saliva of SS patients has been observed in several studies<sup>2,6,7</sup>. From the observed relatively increased salivary antibody levels as compared to serum levels, it has been suggested that these antibodies are synthesized within the salivary glands and that they can be detected in saliva before they become present in the peripheral circulation<sup>7</sup>. Likewise we hypothesised that anti-SS-A or anti-SS-B synthesis within the lacrimal glands of SS patients might result in tear fluid presence of anti-SS-A or anti-SS-B, even in the absence of serum presence of these antibodies.

Furthermore we hypothesized that following prolonged inflammation with accompanying local antibody formation in exocrine glands, the antibody may become present in the peripheral circulation as disease progresses. Previous follow-up studies have not been aimed at anti-SS-A and anti-SS-B seronegative SS patients specifically<sup>8-11</sup>. In general a stable serological profile is observed in the course of SS. However, seroconversion from a negative to positive anti-SS-A or anti-SS-B serological profile was noted in one of these studies in 14% and 16% of SS patients respectively<sup>11</sup>. If confirmed, this would implicate that in some seronegative patients initially not fulfilling the classification criteria for SS, repeated measurement of anti-SS-A and anti-SS-B could result in diagnosing SS on second occasion.

The aims of this study were to assess whether anti-SS-A and anti-SS-B could be detected in tear fluid samples of our seronegative SS patient population, and to examine whether seroconversion to a positive profile occurred after disease had progressed for at least five years since diagnosis.

## METHODS

All patients who were asked to participate gave their informed consent before blood or tear fluid withdrawal was done.

### **Patients, tear fluids and sera**

Tear fluid was collected from 13 anti-SS-A and anti-SS-B seronegative patients fulfilling the EUCG criteria for SS, and 3 anti-SS-A and anti-SS-B seronegative focal sialadenitis patients. Tear fluid samples of 3 anti-SS-A and anti-SS-B seropositive SS patients served as control. Tear fluid was obtained using Schirmer-test paper strips placed in the lower conjunctival sac for 10 minutes. The paper was then stored in 0.5ml phosphate buffered saline (PBS) and immediately stored at – 70° Celsius.

New serum samples taken from 26 patients with sicca symptoms, that were anti-SS-A and anti-SS-B seronegative in the past, were tested for presence of these antibodies. Of these 26 patients 17 fulfilled the EUCG classification criteria for SS. The remaining 9 patients were considered as focal sialadenitis patients (focus score >1, but not fulfilling the UECG criteria). In addition, new serum samples of 16 previously anti-SS-A and anti-SS-B seropositive SS patients, all fulfilling the UECG criteria, were tested. The time interval between initial and second blood sampling ranged between 5 and 12 years.

### **Measurement of anti-SS-A / anti-SS-B in serum by counter-immunoelectrophoresis (CIE)**

Sera obtained after new blood withdrawals were analysed for presence of anti-SS-A and anti-SS-B by CIE, which was also used in the past for analysis of sera obtained at initial diagnosis. For CIE the procedure described by Bunn and Kveder<sup>12</sup> was performed, using bovine spleen extract (BS) for detection of anti-SS-A, and rabbit thymus extract (RT) for detection of anti-SS-B. Plates coated with agarose type L (Behring®) in ENA buffer were used for electrophoresis with the antigen (BS/RT) during 75 minutes, after an initial electrophoresis without antigen during 15 minutes. Plates remained in ENA buffer overnight, and were then transferred to phosphate buffered saline (PBS) for 24 hours. Thereafter the plates were washed once with distilled water (dH<sub>2</sub>O) and then stained with Coomassie blue solution during 4 minutes. After staining the plates were washed again with dH<sub>2</sub>O and drying of the the gel was done using a hair dryer. For confirmation all sera were also tested using the ELISA that was used for tear fluid measurement.

### **Measurement of anti-SS-A and anti-SS-B in tear fluid by ELISA**

Presence of IgG anti-SS-A and IgG anti-SS-B in tear fluid samples was determined by ELISA using Quanta Lite™ SS-A 708570 and Quanta Lite™ SS-B 708575 assays (Inova Diagnostica, Inc. San Diego) respectively, both with anti-IgG conjugate antibodies. The ELISA was performed as recommended by the supplied manufacturers manual. Dilution of tear samples was not performed because the tear samples were already diluted in PBS before storage.

## **RESULTS**

### **Anti-SS-A or anti-SS-B presence in tear fluid**

Very low amounts of tear fluid (approximately 5 µl per sample) were obtained. Despite this, the ELISA showed anti-SS-A presence in the tear fluid of 2 of 13 seronegative SS patients. From these two patients tear fluid was afterwards collected and tested again and this confirmed the presence of anti-SS-A. No anti-

SS-B presence was observed. Both anti-SS-A and anti-SS-B were found in the tear fluid samples of all three seropositive SS patients. Tear fluid samples of seronegative focal sialadenitis patients were negative for both anti-SS-A or anti-SS-B (Table I).

**Table I: Tear fluid presence of anti-SS-A and anti-SS-B in patients with Sjögren's syndrome (SS) and Focal Sialadinitis (FS)**

	n	serum		tear fluid	
		anti-SS-A	anti-SS-B	anti-SS-A	anti-SS-B
SS	3	3	3	3	3
SS	13	0	0	2	0
FS	3	0	0	0	0

(Table shows number of positive serum and tear fluid samples)

### Anti-SS-A and anti-SS-B serological profile

No anti-SS-A or anti-SS-B presence was found in the 26 sera of patients that were previously seronegative (Table II). Serum presence of anti-SS-A and anti-SS-B was confirmed in all 16 previously seropositive SS patients. Taken together, no seroconversion was observed.

**Table II: Serum presence of anti-SS-A and anti-SS-B after at least five years since initial diagnostic measurement**

	n	diagnostic measurement		2 <sup>nd</sup> measurement after > 5 years	
		anti-SS-A	anti-SS-B	anti-SS-A	anti-SS-B
SS	17	0	0	0	0
SS	16	16	16	16	16
FS	9	0	0	0	0

(Table shows number of positive serum samples in CIE as well as ELISA)

## DISCUSSION

SS patients lacking serum presence of anti-SS-A and anti-SS-B are a relatively understudied population in research on SS. However, since exocrinopathy is the most striking phenomenon in SS, an alternative for serological analysis in these patients might be found in evaluating anti-SS-A or anti-SS-B presence in the affected glands or their excreta. In this study tear fluid presence of IgG anti-SS-A was demonstrated and reproduced in 2 of 13 seronegative SS patients. Furthermore tear fluid presence of both anti-SS-A and anti-SS-B was found in seropositive SS patients. Our results support findings of Toker et al.<sup>5</sup> who, also using ELISA, demonstrated IgG anti-SS-A and IgG anti-SS-B in tear fluid of SS patients. However, our study is limited by the small number of evaluated seromegative patients and the small volumes of tear fluid that were obtained. The

latter did not allow tear fluid evaluation using a confirmation technique for ELSIA, such as CIE. Nevertheless, a remarkably low amount of tear fluid (approximately 5 µl) appeared to be enough to detect anti-SS-A by ELISA.

Obtaining larger tear fluid volumes and assessment of IgA and IgM anti-SS-A and anti-SS-B in addition to IgG antibody could result in a higher sensitivity. Further studies should elucidate whether the sensitivity of these antibodies is higher in exocrine gland products than in sera. Since apparently tear presence of anti-SS-A or anti-SS-B is not necessarily associated with serological presence, combined measurement of both body fluids may facilitate clinical diagnosis of SS patients with a negative anti-SS-A and anti-SS-B serological profile.

In this study no conversion from a negative to positive anti-SS-A and anti-SS-B serological profile was found in 17 seronegative SS patients. Although in our hospital the number of anti-SS-A and SS-B seronegative SS patients is low, we evaluated twice as much seronegative SS patients as compared to currently available previous reports<sup>9,10</sup>. In the examined focal sialadenitis patients serum samples also remained negative for anti-SS-A and anti-SS-B. Had this not been the case then these patients would have been diagnosed as having SS according to the EUCG criteria.

In conclusion, this study confirms that anti-SS-A presence can be found in the tear fluid of some seronegative SS patients, and that both anti-SS-A and anti-SS-B can be found in the tear fluid of seropositive SS patients. Furthermore the presence of a stable anti-SS-A and anti-SS-B serological profile in the course of SS was confirmed. Thus, in contrast to repeated serological analysis (which appears of no additional diagnostic value), optimized tear fluid analysis might reveal anti-SS-A or anti-SS-B presence in seronegative SS patients, thereby facilitating the diagnosis of Sjögren's syndrome.

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## CHAPTER 3

# **Anti-alpha-fodrin antibodies do not add much to the diagnosis of Sjögren's syndrome**

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## ABSTRACT

The presence of anti-alpha-fodrin autoantibodies has been reported to be a highly specific and sensitive test for the diagnosis of Sjögren's syndrome (SS). We looked (in Nijmegen) for anti-alpha-fodrin, anti-SS-A60, and anti-SS-B autoantibodies in a cohort of 51 patients with rheumatic diseases (21 primary SS, 6 secondary SS, 12 rheumatoid arthritis (RA), 6 systemic lupus erythematosus (SLE), and 6 scleroderma) and in 28 healthy subjects, using ELISA, immunoblotting, and immunoprecipitation. The same samples were analyzed with an alternative anti-alpha-fodrin ELISA in Hanover. The Nijmegen ELISA of the sera from primary SS showed sensitivities of 43% and 48% for IgA- and IgG-type anti-alpha-fodrin antibodies, respectively. The Hanover ELISA showed sensitivities of 38% and 10% for IgA- and IgG-type anti-alpha-fodrin antibodies, respectively. The ELISAs for alpha-fodrin showed six (Nijmegen) and four (Hanover) anti-alpha-fodrin-positive RA sera. IgA and IgG anti-fodrin antibodies were also present in four patients with secondary SS. The sensitivities of Ro60 and La-antibodies in the Nijmegen ELISA were 67% and 62%, respectively. Unlike anti-alpha-fodrin antibodies, all anti-SS-A60 and anti-SS-B positive sera could be confirmed by immunoblotting or RNA immunoprecipitation. Thus, anti-SS-A and anti-SS-B autoantibodies were more sensitive than anti-alpha-fodrin autoantibodies in ELISA and were more frequently confirmed by other techniques. anti-SS-B antibodies appear to be more disease-specific than anti-alpha-fodrin antibodies, which are also found in RA sera. Therefore, the measurement of anti-alpha-fodrin autoantibodies does not add much to the diagnosis of Sjögren's syndrome.

## INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune exocrinopathy of unknown origin. Therefore the diagnosis of SS, in the absence of a gold standard, is based on criteria containing a number of subjective and objective signs and symptoms. In the past three decades, several sets of criteria have been introduced<sup>1-4</sup>, in which there has been a shift from emphasis on subjective symptoms, such as complaints of dry eyes or dry mouth, towards objective findings. Recently, a widely supported consensus was established to merge the most frequently used European (European Study Group (ESG)) and US (San Diego, San Francisco) classification criteria sets into one US/European set<sup>5</sup>. The authors of all three major classification criteria sets previously used took part in this consensus group. In the US/European classification criteria, more weight is put on the presence of anti-SS-A and anti-SS-B antibodies in the serum, and on the lymphocytic focus score (LFS) of the sublabial glands, both being objective signs. The cutoff point of a positive LFS was set at  $\geq 1.0$ , which ended a long-lasting debate about whether an LFS of  $\geq 1.0$  (ESG criteria),  $> 1.0$  (San Francisco criteria), or  $\geq 2.0$  (San Diego criteria) was most applicable for the diagnosis of SS. This agreement ultimately will produce uniform intercontinental disease prevalence data. However, the disease specificity of particularly anti-SS-B antibodies is limited (besides being found in SS, they are also found in systemic lupus erythematosus (SLE)), and the sensitivities of anti-SS-A and anti-SS-B antibodies range only from 60–75% and 30–50%, respectively<sup>6-9</sup>. Therefore, the search for more sensitive and specific diagnostic markers needs to be continued.

Haneji and co-workers<sup>10</sup> suggested a 120-kDa cleavage product of alpha-fodrin (a cytoskeletal protein) as a candidate autoantigen in SS. They reported that the presence of anti-alpha-fodrin antibodies was very specific for the diagnosis of SS and claimed a very high sensitivity (96%). In another report of the same group, however, these antibodies were also found in some sera of patients with SLE<sup>11</sup>. Their results suggested that anti-alpha-fodrin antibodies might replace anti-SS-A and anti-SS-B antibodies, as a more objective serological marker to improve the diagnostic value of classification criteria. This suggestion was supported by Witte and co-workers, who developed an ELISA for the detection of anti-alpha-fodrin antibodies and showed that IgA antibodies against alpha-fodrin provided an even higher sensitivity than IgG antibodies<sup>12</sup>. The objective of this study was to measure the presence of anti-alpha-fodrin antibodies in the sera of a cohort of patients with well-defined SS at the Department of Rheumatology of the University Medical Center St Radboud, Nijmegen, The Netherlands. A second objective was to evaluate whether positive anti-fodrin ELISA results could be confirmed by at least one alternative biochemical technique such as immunoblotting or protein immunoprecipitation.

## **MATERIALS AND METHODS**

### **Patients and measurement techniques**

The sera of 21 patients (18 women and 3 men, aged 27–76 years, median 55 years) with well-defined primary SS according to the US/European criteria<sup>5</sup> were tested along with the sera of 6 patients with secondary SS (all women, aged 41–55 years), 28 normal healthy subjects (NHS) (19 women and 9 men, aged 24–61 years, median 43 years), 12 patients with rheumatoid arthritis (RA) and without signs of secondary SS (8 women and 4 men, aged 32–72 years, median 47 years), 6 with SLE (all women, aged 33–56 years), and 6 with systemic sclerosis (SSc) (3 women and 3 men, aged 36–49 years). All the patients tested were white. To fulfill the US/European classification criteria, all SS patients had to have a biopsy of the sublabial salivary glands showing an LFS  $\geq 1.0$ . Furthermore, immunohistochemical examination had to show a percentage IgA-containing plasma cells of less than 70, a feature that is also strongly associated with SS and slightly more disease specific than the LFS<sup>13,14</sup>. The comorbidity of the six patients with secondary SS is shown in Table 1. Secondary SS was accompanied by SLE in four patients, by systemic sclerosis in another patient, and by dermatomyositis in yet another. None of the SS patients was receiving an immunosuppressant. The presence of anti-alpha-fodrin antibodies in the sera was measured with three different biochemical techniques: ELISA, immunoblotting with recombinant fodrin, and immunoprecipitation of radiolabeled fodrin. Furthermore, a blinded set of our serum samples was analyzed by Witte and co-workers using the anti-alpha-fodrin ELISA developed in Hanover, Germany (referred to as Hanover ELISA)<sup>12</sup>.

### **Expression of the 120-kDa alpha-fodrin fragment**

For the expression of the antigenic fodrin fragment, we used the alpha-fodrin cDNA in a GST expression vector (pGEX-4T2), which was kindly supplied by Dr Y Hayashi (Tokushima University School of Dentistry, Tokushima, Japan). The cDNA was expressed in BL21 (DE3) cells, and the protein was affinity-purified using glutathione Sepharose beads (Amersham Pharmacia Biotech).

### **ELISA**

The presence of IgA, IgG, and IgM anti-alpha-fodrin antibodies in sera in 100-fold dilution was assessed by ELISA. Plates were coated with purified alpha-fodrin–GST as antigen, and bound antibody was detected essentially as described by Schellekens and colleagues<sup>15</sup>, using rabbit peroxidaseconjugated anti-human immunoglobulins (anti-IgG, anti-IgA, or anti-IgM, DAKO, Glostrup, Denmark). Sera were considered positive when the optical density at 450 nm values after correction for background value exceeded the mean +2 SD of that of a pool of sera from NHS. All ELISAs were performed in duplicate. To check for possible false-positive results because of the presence of the GST moiety in the alpha-fodrin–GST product that was used as the antigen, the ELISA was also performed in the presence of a 10-fold excess of purified carrier GST. anti-SS-B (SS-B) and anti-SS-A (SS-A) autoantibodies were measured by ELISA, using recombinant La and Ro60 proteins. All sera were also analyzed by immunoblotting and RNA immunoprecipitation to confirm the presence of anti-SS-A and anti-SS-B antibodies, as previously described<sup>16</sup>.

### **Immunoblotting**

To confirm the ELISA results, reactivity of sera against recombinant fodrin–GST was evaluated by western blotting essentially as described elsewhere<sup>17</sup>. The human sera were diluted 5000-fold with blocking buffer. A second antibody directed against total human immunoglobulin was used (DAKO, Glostrup, Denmark). In a similar type of experiment, immunoblots containing extracts from apoptotic Jurkat cells, prepared according to the method of Zampieri and colleagues<sup>18</sup> and believed to contain the native and possibly modified apoptotic alpha-fodrin fragment of 120 kDa described by Haneji and coworkers, were used. Visualization was performed by chemiluminescence.

### **Immunoprecipitation of radiolabeled alpha-fodrin**

For labeling of cellular proteins, HeLa cells were incubated in medium without methionine and supplied with 2% dialyzed fetal calf serum (FCS). After 1 hour, 10  $\mu$ Ci <sup>35</sup>S-methionine per ml was added and the concentration of dialyzed FCS was raised to 5%. After an additional 4 hours, 1 volume of complete medium containing 10% FCS (undialyzed) was added and incubation was continued for 16 hours at 37°C. The cells were collected by centrifugation, washed, and homogenized in lysis buffer (50mM Tris/HCl, pH 7.5, 0.5% NP40, 100mM KCl, 1mM dithioerythritol, 1mM EDTA) containing a mixture of protease inhibitors. IgG antibodies from sera to be analyzed (SS and controls) were coupled to protein A-agarose beads (Biozym, Landgraaf, The Netherlands) and immunoprecipitations were carried out as described by Raijmakers and colleagues<sup>17</sup>.

### **Hanover ELISA confirmation**

Another way to confirm our ELISA results was to compare our data with those obtained with the Hanover ELISA developed by Witte and colleagues<sup>12</sup>. A blinded set serum samples was therefore analyzed in Hanover.

## **RESULTS**

### **Analysis for anti-alpha-fodrin, anti-SS-B, and anti-SS-A by ELISA**

Using the purified alpha-fodrin–GST protein encoded by the cDNA construct obtained from Dr Hayashi, we developed an ELISA (hereafter referred to as the Nijmegen ELISA) that was used for the analysis of SS and control sera. The Nijmegen IgA ELISA test appeared to be reasonably specific. Only one serum positive for anti-alpha-fodrin was found among the 12 RA sera. In the 28 NHS sera and 12 sera from SLE and SSc patients, no IgA anti-alpha-fodrin antibodies were detected. In the IgG ELISA, however, six RA sera were positive; the other control sera (NHS, SLE, and SSc) were negative. Of the 21 sera from primary SS, 10 were found positive the IgG ELISA, indicating a disease sensitivity of IgG antibodies against alpha-fodrin of 48%. Nine (43%) of the 21 primary SS sera were found to contain IgA antibodies against alpha-fodrin (Table 1) and 3 (14%) of the 21 contained IgM antibodies against alpha-fodrin (not shown). Five of the nine primary SS sera with IgA antibodies to fodrin also contained IgG antibodies directed against this antigen. To be sure that the antibodies measured were directed against the fodrin part of the fodrin–GST fusion protein, the ELISAs were also carried out in the presence of 10-fold excess of purified GST protein. Essentially the same results

were obtained. The same sera (blinded) were also analyzed in Hanover by Witte and coworkers using their alpha-fodrin ELISA [12]. The Hanover results showed a disease sensitivity of 38% for IgA and of 10% for IgG antibodies against alpha-fodrin. Six 8 sera from primary SS that were positive in the Hanover IgA ELISA were also positive in the Nijmegen ELISA, so that 6 of 21 sera from primary SS (29%) conclusively seemed to contain IgA antibodies directed against alpha-fodrin. While the data for IgA-positive sera from both ELISAs were quite congruent, data for IgG-positive sera showed clear discrepancies (Table 1). We do not know why. We also looked for the classic anti-SS-B and anti-SS-A autoantibodies in the 21 sera from primary SS. Fourteen sera contained anti-SS-A antibodies (sensitivity 67%) and 13 sera contained anti-SS-B antibodies sensitivity 62%). These activities were confirmed by least one other technique (immunoblotting and/or precipitation). anti-SS-B and anti-SS-A activities were absent all control sera that were included in this study (data shown). There was also considerable overlap between presence of anti-alpha-fodrin and anti-SS-A or anti-SS-B. Of IgA-positive sera from primary SS, 6 contained anti-SS-A and 5 contained anti-SS-B, while of the 10 IgG-positive sera, all contained anti-SS-A also and 7 contained anti-SS-B antibodies (Table 1). Four of six sera from patients with secondary SS contained IgA and IgG antibodies against alpha-fodrin. Three sera also contained anti-SS-A and anti-SS-B antibodies (Table 1). From these results we conclude that alpha-fodrin antibodies are present in SS sera and that majority of these antibodies are of the IgG and IgA class, but that their frequency in SS sera is not higher than of the classic autoantibodies directed against the Ro60 and La antigens.

### **Analysis for anti-alpha-fodrin antibodies by western blotting and immunoprecipitation**

We also analyzed the sera for anti-alpha-fodrin antibodies by two other techniques (immunoblotting and protein immunoprecipitation) to confirm the ELISA results. In general, these two techniques appeared to be less suited for this purpose. The size of the antigen (250 kDa) and limited presence in cultured cells precluded both efficient blotting and efficient labeling by <sup>35</sup>S-methionine. There were also some background problems that in some cases made it difficult to distinguish between positive and negative sera. We therefore decided to count only those that were clearly positive. Of the sera from primary SS, five appeared to be positive immunoblotting and four precipitated a protein with expected molecular weight of fodrin. Two of those immunoprecipitated fodrin were also positive immunoblot, and three also contained IgA and/or IgG antibodies to alpha-fodrin as measured by ELISA (Table 1). The presence of anti-fodrin antibodies in four sera patients with secondary SS was confirmed immunoblotting (four of four) and by immunoprecipitation (three of four) (Table 1). The use of apoptotic extracts (to increase amount of antigenic fodrin cleavage product) did in our hands not improve the suitability of these techniques for the detection of anti-alpha-fodrin antibodies (data not shown).



**Table 1 Autoantibodies in Nijmegen sera from a cohort of patients with Sjögren's syndrome**

Pt no.	IgA <sup>a</sup>		IgG <sup>b</sup>		IB <sup>d</sup>	IP <sup>e</sup>	Ro60 <sup>f</sup>	La <sup>g</sup>	LFS <sup>h</sup>	Comorbidity
	Nijmegen	Hanover <sup>c</sup>	Nijmegen	Hanover <sup>c</sup>						
Primary Sjögren's syndrome										
1	+	+	+	-	-	-	+	-	+	
2	-	-	-	-	-	-	-	+	+	
3	+	-	-	-	-	-	-	-	+	
4	+	+	+	-	-	-	+	-	+	
5	+	+	+	-	+	+	+	+	+	
6	+	-	-	-	-	+	+	+	+	
7	+	-	-	-	-	-	-	-	+	
8	-	+	-	-	-	-	-	-	+	
9	-	+	+	-	-	-	+	-	+	
10	+	+	+	-	-	+	+	+	+	
11	-	-	-	-	-	-	+	+	+	
12	-	-	-	-	+	-	+	-	+	
13	-	-	+	-	-	-	+	+	+	
14	-	-	-	-	+	+	+	+	+	
15	-	-	+	-	-	-	+	+	+	
16	-	-	-	-	+	-	-	-	+	
17	-	-	+	-	-	-	+	+	+	
18	+	+	-	-	-	-	-	+	+	
19	-	-	-	-	+	-	-	-	+	
20	+	+	+	+	-	-	+	+	+	
21	-	-	+	+	-	-	+	+	+	
Secondary Sjögren's syndrome										
22	+	+	+	-	+	+	+	+	+	SLE
23	+	+	+	+	+	+	+	+	+	SLE
24	-	-	-	-	-	-	+	-	+	SLE
25	+	+	+	+	+	+	-	-	+	SLE
26	+	+	+	-	+	-	+	+	+	SSc
27	-	-	+	-	-	-	+	-	+	DM

<sup>a</sup>IgA, ELISA measuring presence of IgA antibodies directed against  $\alpha$ -fodrin; <sup>b</sup>IgG, ELISA measuring presence of IgG antibodies directed against  $\alpha$ -fodrin; <sup>c</sup>findings in Hanover for the sera originally tested in Nijmegen; <sup>d</sup>IB, immunoblotting results ( $\alpha$ -fodrin); <sup>e</sup>IP, protein immunoprecipitation results ( $\alpha$ -fodrin); <sup>f</sup>Ro60, ELISA measuring presence of antibodies directed against Ro60-antigen; <sup>g</sup>La, ELISA measuring presence of antibodies directed against La-antigen; <sup>h</sup>LFS, lymphocytic focus score  $\geq 1.0$  in sublabial minor salivary glands biopsy. DM, dermatomyositis; Pt, patient; SSc, systemic sclerosis; SLE, systemic lupus erythematosus.

## DISCUSSION

The presence of anti-SS-A and anti-SS-B autoantibodies has been part of Sjögren's syndrome (SS) classification criteria including the recently established US-European consensus group criteria, in which they play a more significant role than before<sup>5</sup>. In the US/Eur classification criteria the presence of either anti-SS-A or anti-SS-B autoantibodies or a positive salivary gland biopsy (LFS $\geq$ 1.0) is mandatory for the classification of SS. The disease sensitivity of anti-SS-A and anti-SS-B autoantibodies has been reported to be 60-75 % and 30-50 % respectively<sup>6-9</sup>, whilst disease specificity of particularly anti-SS-B autoantibodies is generally considered as reasonably high. anti-SS-B autoantibodies are mostly found in SS and SLE patients, and rarely in other diseases or normal healthy subjects. Nevertheless, there is certainly a need for a SS specific autoantibody showing a better disease sensitivity and specificity profile.

Haneji et al. suggested the presence of anti- $\alpha$ -fodrin autoantibodies as a highly specific diagnostic marker for SS. In their initial paper they reported that 96% of primary SS sera reacted with  $\alpha$ -fodrin<sup>10</sup>. In follow-up studies however, they also noticed the presence of these autoantibodies in SLE patients<sup>11</sup>. Witte and collaborators showed a much lower disease sensitivity of 64% and 47% in primary and secondary Sjögren's syndrome, respectively, when focussing on IgA antibodies against  $\alpha$ -fodrin rather than IgG antibodies<sup>12</sup>. Their data suggest a sensitivity similar to that of anti-SS-A antibodies. However, Witte and co-workers also noticed two positive sera in RA patients without sicca symptoms, and one positive serum in the SLE group. These results indicate that also the disease specificity of anti- $\alpha$ -fodrin antibody might be lower than reported previously. In our cohort of primary SS patients, the ELISA tests of 21 sera showed a sensitivity of 43% for IgA antibodies, and 48% for IgG antibodies against  $\alpha$ -fodrin which is comparable to the percentages reported by Witte and co-workers. In the blinded set of control sera analyzed in Nijmegen and Hannover, no positives were found in the NHS, SLE or SSc sera, suggesting that the ELISA tests in both laboratories are specific. However, of the 12 RA sera tested in Hannover, 4 RA sera came out as positive in the IgA-ELISA, including the one RA serum that was also positive in Nijmegen. Six RA sera were positive in the IgG-ELISA in Nijmegen; 3 of them also in the IgG ELISA of Hannover. These data, together with the previously reported positive RA sera by Witte and co-workers, indicate that the presence of anti-fodrin antibodies in a significant number of RA patients can not be ruled out. Although in this study disease specificity was not evaluated against a large variety of disease control sera, these results suggest that the disease specificity of anti-fodrin antibodies is unlikely to exceed that of anti-SS-B antibodies which are almost exclusively found in either SS or SLE sera. This study also showed that there are discrepancies between the two anti-fodrin ELISA systems. An explanation of this imperfect reproducibility between the two laboratories might be that the titers of anti- $\alpha$ -fodrin antibodies in patient sera were generally low in both ELISA systems. This makes that small changes in the protocol become important for the outcome. These

observations underline once more the importance of an easy-to-perform alternative biochemical technique to confirm ELISA data<sup>19</sup>. Based on the difficulties encountered in this study to confirm the presence of anti-fodrin antibody via alternative techniques, it is questionable whether anti-fodrin antibodies should substitute for the classical anti-SS-A and anti-SS-B antibodies in the classification criteria of SS. All anti-SS-A and anti-SS-B activities in our sera detected by ELISA could be confirmed using alternative biochemical techniques. Besides that, the observed sensitivity of these classic autoantibodies is higher than that of anti- $\alpha$ -fodrin antibodies, regardless of the anti-fodrin ELISA system (Hannover versus Nijmegen ELISA) that was used. In addition, a considerable overlap between the presence of anti-SS-A/anti-SS-B antibody and anti- $\alpha$  fodrin antibody was observed. A potential contributing role for anti-fodrin antibody measurement to detect SS patients that are negative for anti-SS-A and anti-SS-B autoantibodies appears therefore unlikely.

Based on the lower frequency, as compared to anti-SS-A and anti-SS-B, and the questionable specificity, we conclude that testing for anti- $\alpha$ -fodrin antibodies does not have much additional value for the diagnosis of Sjögren's syndrome.

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## CHAPTER 4

# **Estrogen-beta receptor expression in skin, salivary glands and salivary gland infiltrating lymphocytes in Sjögren's syndrome patients**

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(submitted)

## ABSTRACT

Sjögren's syndrome (SS) is considered an autoimmune exocrinopathy of unknown origin, predominantly affecting salivary and lacrimal glands. Cutaneous exocrinopathy is presumed to account for the frequently reported xerosis in SS. Although SS predominantly affects women, no direct estrogen mediated pathophysiological mechanisms have been identified in previous studies. Estrogen-beta (ER $\beta$ ) receptor is the predominant ER in normal skin and salivary glands and has not yet been evaluated in SS patients specifically. The aims of this study were to explore skin biopsy specimens of SS patients for signs of exocrinopathy and to describe estrogen-alpha (ER $\alpha$ ) and estrogen-beta (ER $\beta$ ) receptor expression in the skin and salivary glands of SS patients as compared to that of normal healthy subjects (NHS). Sublabial minor salivary gland biopsy specimens and skin biopsy specimens of 17 SS patients and 5 NHS were examined for presence of lymphocytic infiltrates and immunohistologically analysed for ER $\alpha$  and ER $\beta$  expression. All SS patients (12 primary, 5 secondary SS) fulfilled the American-European Consensus Group classification criteria for SS. ER $\alpha$  expression was assessed using the 1D5 monoclonal antibody (Dakocytomation, High Wycombe, UK); for ER $\beta$  detection the 14C8 monoclonal antibody (Genetex, AbCam, Cambridge, UK) was used. None of the 17 SS skin samples showed signs of inflammatory exocrinopathy. In both skin and salivary gland samples ER $\beta$  was the predominant ER subtype. In skin samples ER expression did not differ between NHS and SS patients. In the salivary gland samples of all NHS and 8 SS patients a focal weak nuclear staining of ER $\beta$  was observed. However, diffuse intense nuclear staining (n=4) and focal predominantly cytoplasmatic staining (n=5) were also observed in SS samples. Interestingly in 5 SS patients some infiltrating lymphocytes in the salivary gland tissue also stained positive for ER $\beta$ . ER $\alpha$  was poorly expressed in all examined tissues. Evidence for inflammatory cutaneous exocrinopathy in SS was not found. In SS patients ER $\beta$  is the predominant ER in skin and salivary gland tissue. The observed differentiated ER $\beta$  expression and the presence of ER $\beta$  positive lymphocytes within the salivary gland infiltrates in SS patients may reflect a role for ER $\beta$  in the pathogenesis or maintenance of SS.



## INTRODUCTION

Sjögren's syndrome (SS) is considered a chronic autoimmune exocrinopathy, mainly involving salivary and lacrimal glands, but also affecting other exocrine glands e.g. in the upper airways, the vagina and possibly in the skin. SS is clinically characterised by sicca symptoms such as dry eyes and mouth. Infiltrating lymphocyte aggregates seen in biopsy specimens of sublabial minor salivary glands, form one of the hallmarks in the current American-European Consensus Group (AECG) classification criteria for Sjögren's syndrome<sup>1</sup>. Similar histological characteristics have also been observed in lacrimal gland tissue<sup>2</sup>. Although the majority of sublabial salivary gland biopsy procedures are without complications post-biopsy neuropathy reflected by localised numbness can occur<sup>3</sup>. Also some patients may feel uncomfortable towards intraoral invasive procedures. Theoretically a diagnostic skin biopsy might serve as an alternative to salivary gland tissue biopsy in SS patients if similar histopathological findings could be found in cutaneous exocrine glands. Since xerosis (dry skin) is frequently reported in SS, an underlying cutaneous exocrinopathy, similar to salivary and lacrimal gland tissue exocrinopathy, is presumed in textbooks of rheumatology<sup>4</sup>. However, in contrast to salivary gland tissue, few studies have been conducted regarding histological changes in the skin of SS patients. Perivascular infiltrates reflecting some degree of vasculitis as a systemic manifestation of SS are the most frequently reported histopathological finding in the skin of SS patients. Pure cutaneous exocrinopathy reflected by periglandular lymphocytic infiltrates, such as seen in salivary gland tissue, has only been documented in a few case reports published over the last decennia<sup>5-10</sup>. Cutaneous exocrinopathy could be located in sebaceous glands as well as eccrine or merocrine (formerly known as apocrine) sweat glands. Merocrine sweat glands, present in the axillae and anogenital area, are known to be under the control of sex hormones. More recently presence of sex steroid receptors has also been noticed in eccrine sweat glands<sup>11</sup>.

The sebaceous glands constitute the other exocrine component of human skin. The sebum, rather than sweat, controls moisture loss from the epidermis. Decreased sebaceous gland function is therefore reflected in xerosis which might also be a constituent of the sicca complex in SS. Estrogens have a profound effect on sebaceous gland function which is opposite to that of androgens. In both sexes, estrogen administration decreases the size of the sebaceous glands and the production of sebum. Thus, sex steroids are clearly involved in cutaneous exocrine gland functioning (both sweat and sebaceous glands). The tissue-specific distribution of sex steroid receptors has recently been further explored following the discovery of a second type of estrogen receptor (ER)<sup>12</sup>. A series of reports on different localizations and functioning of the two distinct subtypes, ER $\alpha$  and ER $\beta$ , in human tissues has meanwhile become available. ER $\beta$  appears to be the predominant ER in skin, salivary glands, lacrimal glands and oral epithelium<sup>11,13,14</sup>. In literature these tissues are referred to as 'non-classic targets' because sex steroids are thought to act on the reproductive organs. However, it should be noted that in SS the same tissues constitute the disease-specific target organs in which exocrinopathy becomes

manifest. Since Sjögren's syndrome, like e.g. SLE, is a disorder predominantly affecting women, a role for sex steroids in the pathogenesis has been assumed before. Thus, the tissue-specific distribution of sex steroid receptors might provide clues to the mechanism of exocrinopathy in SS patients. Kassi and colleagues recently reported presence of ER $\alpha$  and splicing variants of ER $\alpha$  in cultured epithelial cells from salivary glands of SS patients, but also in normal health subjects (NHS) (which has been previously described). ER $\beta$  presence was reportedly not evaluated in their study<sup>15</sup>. To our knowledge the expression of both ER $\alpha$  and ER $\beta$  receptor in skin and salivary gland tissue has not yet been studied in SS patients specifically.

This study was aimed to examine skin biopsy specimens of SS patients for signs of cutaneous exocrinopathy and to describe the expression of ER $\alpha$  and ER $\beta$  in skin and salivary gland biopsy specimens of SS patients. We hypothesized that a distinct expression pattern of ER $\beta$  receptors in skin or salivary gland tissue of SS patients as compared to NHS might be present and if so, might attribute to decreased exocrine gland function in SS patients.

## **PATIENTS AND METHODS**

### **Patients and biopsy sampling**

After informed consent, excision biopsy was performed in the lower back of 17 SS patients, all fulfilling the AECG classification criteria<sup>1</sup>. Lower back excision biopsy was chosen in order to obtain skin biopsy specimens containing a sufficient amount of sebaceous as well as sweat glands (located deep in the dermis). Previously taken salivary gland biopsies of the same patients as well as skin and salivary gland samples of 5 normal healthy subjects (NHS) were also included for ER subtype expression analysis. A total of 12 patients had primary SS, whereas 5 had overlap syndromes (2 with SS/systemic sclerosis, 2 with SS/systemic lupus erythematosus, 1 with SS/rheumatoid arthritis)(Table 1). From all paraffin-embedded biopsy specimens eight serial sections (4  $\mu$ m) were examined for presence of lymphocytic infiltrates. The biopsy specimens were then further processed to evaluate ER $\alpha$  and ER $\beta$  presence as described below.

### **Antibodies and Immunohistochemistry**

Serial sections (4  $\mu$ m) were mounted on Superfrost slides and dewaxed. The sections were blocked for endogenous peroxidase activity with 0,3% H<sub>2</sub>O<sub>2</sub>/PBS for 30min. Sections were then subjected to heat-mediated antigen retrieval in 0.01M citrate buffer, pH6.0, for 13min (ER $\alpha$ ) at 750w in microwave. Sections were allowed to cool undisturbed to approximately 37 degrees Celsius over the next 20min, washed (5min each) in PBS. After cooling whilst in the buffer solution (20 min) sections were washed in PBS. The sections were then incubated with the established 1D5 monoclonal antibody diluted 1:200 (ER $\alpha$ , Dakocytomation, High Wycombe, UK) and 14C8 monoclonal antibody diluted 1:100 (ER $\beta$  Genetex, AbCam, Cambridge, UK) in PBS for 30min. Sections were then washed in PBS. Bound antibodies were visualized using Powervision (ready to use, Immunologic, 30 min) and, after washing again in PBS, Powervision diaminobenzadine (DAB) plus (Immunologic, 5 min). After washing the sections again in PBS nuclear staining was performed using hematoxyline (1 min) followed blue-dying in by running tap water for 10 min. Sections were then dehydrated

and permanently mounted using DEPEX mounting medium. Female breast tissue samples were used as an external control for both antibodies.

**TABLE 1 : a selection of patients characteristics grouped by ER $\beta$  staining pattern types**

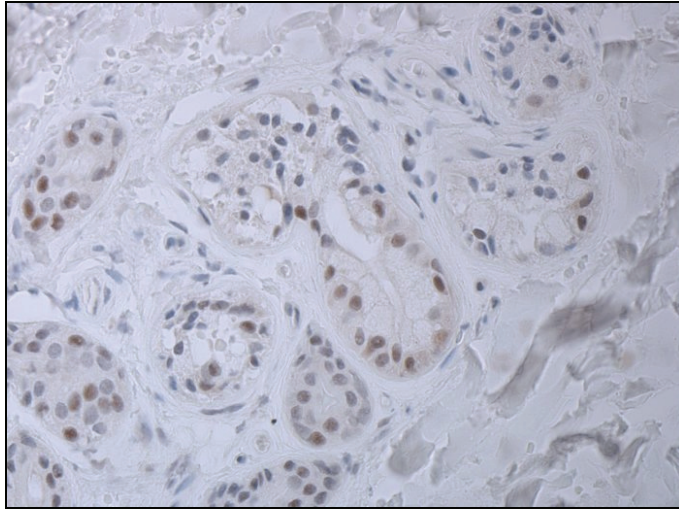
staining type	n	pSS/sSS	SS-A	SS-B	RF	igG	note
<i>diffuse intense nuclear</i>							
	1	pSS	+	+	-	+	polyneuropathy
	2	pSS	+	+	+	+	Hashimoto thyroiditis
	3	pSS	-	-	+	-	Klinefelter's, cerebral vasculitis
	4	SS/SLE	+	+	-	+	serositis
<i>focal cytoplasmatic</i>							
	1	pSS	+	+	+	+	
	2	pSS	+	+	+	+	
	3	pSS	+	+	+	-	
	4	SS/SLE	+	+	+	+	
	5	SS/SSc	-	-	+	-	
<i>focal weak nuclear</i>							
	1	pSS	-	-	+	-	
	2	pSS	-	-	+	-	
	3	pSS	-	-	+	+	
	4	pSS	-	+	+	+	
	5	pSS	-	-	+	-	
	6	pSS	+	+	+	-	
	7	SS/SSc	+	+	-	+	
	8	SS/RA	-	-	+	+	

## RESULTS

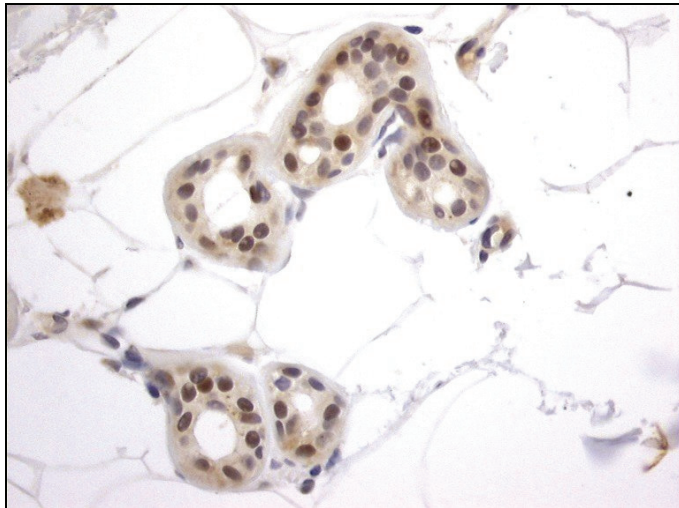
### Skin biopsy specimens

No mononuclear cell infiltrates were seen in serial sections of paraffin-embedded skin biopsy samples of SS patients or NHS. In both SS and NHS skin samples ER $\alpha$  was poorly expressed, being restricted to staining of some sebocytes in the basal layer of sebaceous glands. Furthermore in eccrine sweat glands minimal staining for ER $\alpha$  was only observed in two SS skin samples. In contrast, ER $\beta$  was much more widely expressed throughout the different skin layers. Nuclear ER $\beta$  staining was found in all layers of the epidermis as well as in eccrine sweat glands and some hair follicles. The ER $\beta$  staining pattern in SS skin tissue, which varied in intensity from weak to strong, did not differ from that of NHS samples (Figure 1A, 1B).

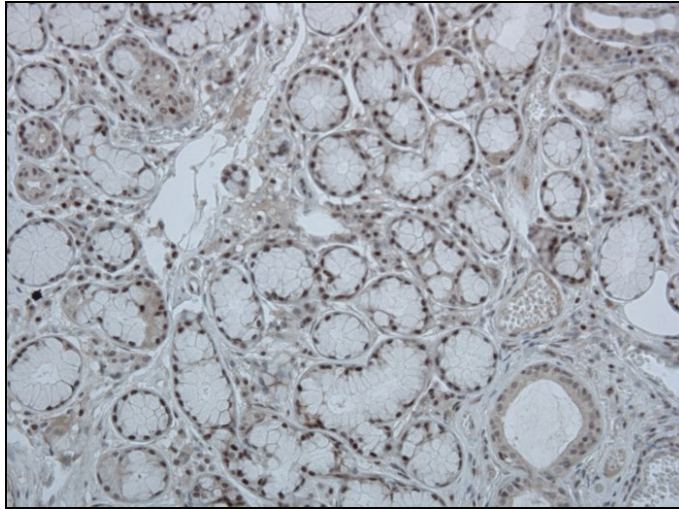
**FIGURE 1A -- SKIN – SJÖGREN'S SYNDROME**



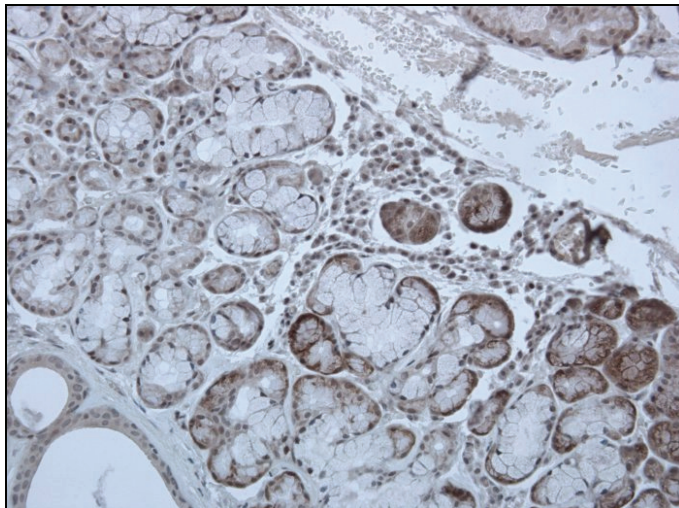
**FIGURE 1B -- SKIN – NORMAL HEALTHY SUBJECT**



**FIGURE 1C -- SALIVARY GLAND – SJÖGREN'S SYNDROME**  
diffuse intense nuclear ER-beta staining



**FIGURE 1D -- SALIVARY GLAND – SJÖGREN'S SYNDROME**  
focal weak cytoplasmic ER-beta staining





### **Sublabial minor salivary gland biopsy specimens**

Previous diagnostic findings showed a focus score  $> 1$ , i.e. more than one mononuclear cell infiltrate containing at least 50 inflammatory cells per  $4 \text{ mm}^2$ , in all salivary gland samples of SS patients. In contrast, the focus score of NHS samples was  $< 1.0$  (range 0-0-4). Furthermore, previously performed immunohistochemical examination of these samples showed a percentage IgA containing plasma cells  $< 70$  in SS patients only, which underlined that these were well defined SS patients<sup>16,17</sup>.

In concordance with the findings in skin tissue, ER $\alpha$  was poorly expressed in salivary gland samples, being restricted to nuclear staining of a few ductal cells. Again, the ER $\beta$  subtype was much more widely expressed. Three different ER $\beta$  staining patterns in terms of distribution, intracellular localization and staining intensity, were observed in SS salivary gland samples. The samples of 4 SS patients showed a diffuse and intense nuclear staining in sero-mucous units (Figure 1C), whereas samples of 5 other SS patients showed a focal, predominantly cytoplasmatic staining in sero-mucous units (Figure 1D). The remaining 8 samples of SS patients showed a pattern comparable to the pattern observed in NHS, with focal weak nuclear staining of sero-mucous units (Figure 1E). No commonly shared clinical or serological features were identified in those patients that showed the same type of ER $\beta$  staining pattern. Also the ER $\beta$  staining pattern was not sex-related. The distinct staining patterns of sero-mucous units was accompanied by sporadic weak staining of ductal cells in all examined specimens. Interestingly we coincidentally noted that in the samples of 5 SS patients staining for ER $\beta$  was also positive in 10-30% of the lymphocytes present within plasmalymphocytic infiltrates (Figure 1F). We did not identify a commonly shared clinical feature in those patients that showed a markedly increased nuclear ER $\beta$  staining pattern or ER $\beta$  positive lymphocytes.

### **DISCUSSION**

In this study a predominance of ER $\beta$  expression, as compared to ER $\alpha$ , was found in skin and salivary gland biopsy samples of SS patients. This is in line with the previously described predominance of ER $\beta$  in skin and salivary glands of NHS. Skin samples of both NHS and SS patients showed similar ER $\alpha$  and ER $\beta$  staining patterns and a total absence of periglandular lymphocytic infiltrates. By examining eight serial sections of each biopsy specimen the risk of missing an infiltrate (sampling error) was minimized. Thus, in concordance with previous study results, a skin biopsy does not appear to provide an alternative for salivary gland biopsy in the diagnosis of Sjögren's syndrome. Based on these study results, (immuno)histological evidence for the presence of cutaneous exocrinopathy in SS is still lacking. Therefore, from a pathophysiological point-of-view, xerosis (dry skin) can currently not be considered a constituent of the sicca complex in SS.

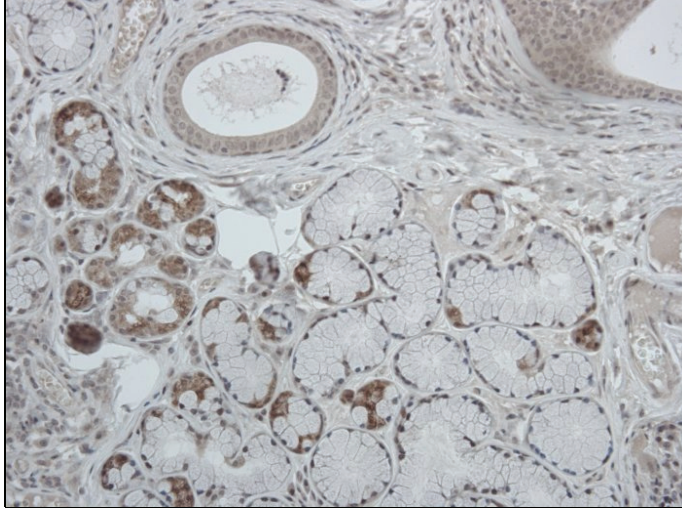
In salivary gland specimens of SS patients three distinct ER $\beta$  staining patterns were noticed. These staining patterns differed from each other in intensity as well as in

localization of ER $\beta$  staining. One of these three patterns was similar to that of NHS. To our knowledge, although ER $\beta$  positive lymphocytes have recently been reported in human secondary lymphoid tissues<sup>18</sup>, the observation of ER $\beta$  positive lymphocytes within the focal sialadenitis infiltrates in SS patients has not been described before. It will be challenging to further elucidate the function and relevance of the observed ER $\beta$  expression patterns and ER $\beta$  positive lymphocytes in salivary gland tissue of SS patients. For this purpose selective estrogen receptor modulators (SERMs) may offer an interesting study tool.

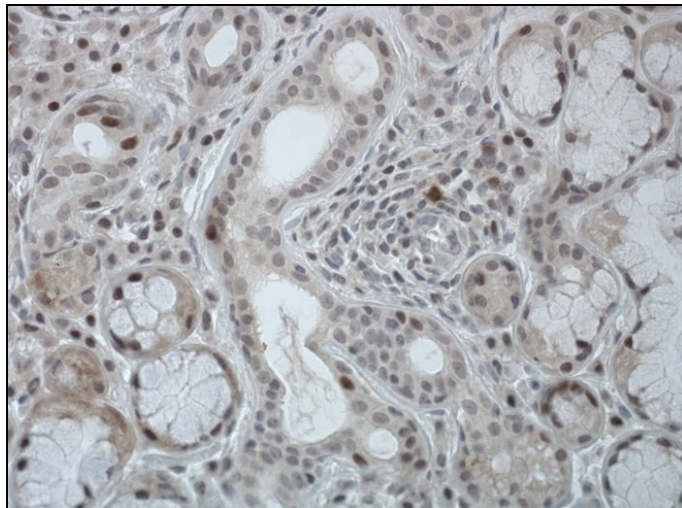
Since Sjögren's syndrome, like e.g. SLE, is a disorder predominantly affecting women, a role for sex steroids in the pathogenesis has been assumed before. Reports of both beneficial and adverse effects of estrogen-replacement therapy on the course of SS or SLE provide additional support for this hypothesis. Interestingly, estrogen activity has also been linked to cutaneous expression of the classical Sjögren's syndrome related antibodies anti-SS-A and anti-SS-B, which are also found in SLE patients. Estradiol has been reported to enhance binding of antibodies specific for SS-A and SS-B to cultured human keratinocytes<sup>19-23</sup>. It must be noted that all studies were performed *in vitro* in an experimental setting using cultured cells.

Estrogens have been reported to exert complex tissue-specific effects<sup>24</sup>. The same stimulus thereby leads to a different response in ER $\alpha$  cells compared to ER $\beta$  cells. ER $\alpha$  is almost always an activator, whereas ER $\beta$  can inhibit the action of ER $\alpha$  by forming a heterodimer with it<sup>25</sup>. Moreover, microarray analysis in mice with deletions of ER $\alpha$  or ER $\beta$  has shown that ER $\beta$  can inhibit the transcription of 240 estrogen-responsive genes by 46 percent<sup>26</sup>. The relative levels of expression of these two receptor isoforms will therefore affect the cellular responsiveness to estrogens<sup>27,28</sup>. Thus, the observed variations in ER $\beta$  expression within SS patients might offer an explanation for the conflicting data of estrogen-replacement-therapy in SS<sup>29,30,31</sup>. ER $\beta$  may hypothetically also act as an anti-androgen, thereby supporting a hypothesis put forward by others that women with SS may be androgen deficient<sup>32</sup>. In conclusion in this study no (immuno)histological evidence was found to support the hypothesis that xerosis in SS patient results from an underlying cutaneous exocrinopathy. Furthermore, we have demonstrated that ER $\beta$  is the predominant ER in salivary gland and skin tissue of SS patients, which is in concordance with previous findings in NHS. However, in SS patients ER $\beta$  expression in salivary glands was found to differ significantly from NHS and between patients. In addition, in SS patients ER $\beta$ -positive infiltrating lymphocytes can be found in the characteristic focal sialadenitis lesions. Further studies should point out whether this differentiated ER $\beta$  expression is of pathophysiological relevance. If so, new pathophysiological and therapeutic concepts for SS may be developed by using SERMs.

**FIGURE 1E -- SALIVARY GLAND – SJÖGREN'S SYNDROME**  
focal weak nuclear ER-beta staining



**FIGURE 1F -- SALIVARY GLAND – SJÖGREN'S SYNDROME**  
ER-beta staining of infiltrating lymphocytes





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## CHAPTER 5

# **The synergistic value of focus score and IgA% score of sublabial salivary gland biopsy for the accuracy of the diagnosis of Sjögren's syndrome: A 10-year comparison**

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## ABSTRACT

### **Objective:**

Increasing the accuracy of the diagnosis of Sjögren's syndrome (SS) by putting emphasis on objective findings such as presence of Ro- and La autoantibodies and abnormal salivary gland tissue (SGT) histology is a current issue. In order to obtain optimal disease-sensitivity and specificity of SGT findings histological and immunohistological SGT examination were compared. The first describes the extent of the lymphocytic infiltrate as a focus-score (LFS), whereas the latter describes the composition of the infiltrate as a percentage IgA-containing plasmacells (IgA%).

### **Methods:**

Both LFS and IgA% scores were assessed in 279 SGT biopsies taken of patients with symptoms suggestive for SS. In case histological conclusions did not match with immunohistological conclusions patients were assigned to so-called mismatch groups. Patients in the mismatch groups were further classified using objective, serological parameters (RF, anti-SS-A, anti-SS-B, ANA, gammaglobulin level).

### **Results:**

In 249 samples (89%) LFS and IgA% resulted in the same conclusion. Within this group a total of 63 SGT samples (25%) were characteristic for SS showing LFS >1.0 and IgA% <70. In the mismatch groups after serological classification both false positive as well as false negative scores were observed less frequently for IgA% as compared to LFS (50% versus 75% and 25% versus 50% respectively).

### **Conclusions:**

Additional immunohistological SGT examination provides higher disease sensitivity and specificity than histological SGT examination alone, thereby increasing accuracy of SS diagnosis.

## INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune exocrinopathy of unknown origin. Therefore the diagnosis of SS, in the absence of a golden standard is based on criteria containing a number of subjective and objective symptoms and signs. In the past three decades several sets of criteria have been introduced<sup>1-4</sup>. In recent criteria sets it is noteworthy that more weight is put on objective than subjective findings such as dry eyes or dry mouth. This shift towards objective findings is partly based on the fact that in an era of computers and dry working places the frequency of dry eyes or dry mouth complaints increases. As these symptoms are part of the currently used criteria, the number of false-positives increases. When the presence of anti-SS-A- and anti-SS-B autoantibodies, as well as on a sublabial salivary gland tissue (SGT) biopsy focus-score (LFS) >1 is emphasised, by requiring presence of both of these objective pathophysiological signs, the number of false-diagnosed SS patients will reduce considerably. If the European Study Group (ESG) classification criteria<sup>1</sup> are modified in this way the prevalence of SS in European countries is expected to lower from 1-3% to about 0.5 %. The prevalence of SS in Europe would then be similar to the proportion diagnosed with San Diego or San Francisco criteria applied in the US<sup>5</sup>. However, when a greater value is attributed to SS-A- and SS-B-autoantibodies and SGT biopsy, the disease-sensitivity and disease-specificity of each of these objective parameters becomes more important and should be reconsidered. The focus score, based on the extent of the lymphocytic infiltrate in SGT biopsy, is the most used objective target-organ specific sign in the diagnosis of SS. It is also the only histological parameter named in the current classification criteria. It is generally considered that a focus score >1 is strongly associated with SS. But meanwhile several reports have shown that a focus score >1 can also be found in other systemic disease (e.g. rheumatoid arthritis (RA), lupus erythematoses disseminatus, primary biliary cirrhosis, AIDS, myasthenia gravis, graft-versus-host disease) as well as in 5-10% of normal healthy subjects, thereby reducing its disease-specificity for Sjögren's syndrome<sup>6-8</sup>. More recently it was shown that a potential negative influence of external factors like smoking and use of medication on the number of foci cannot be ruled out, thereby reducing the sensitivity of the focus score<sup>9,10</sup>. Therefore additional examination of the SGT might help to differentiate between true- and false positive and negative focus scores. One of the methods to do so is quantitative immunohistological (QIH) examination of the SGT biopsy. Here the composition of the lymphocytic infiltrate is described in terms of immunoglobulin subtypes (IgG, IgM, and IgA). In the QIH criterion introduced in 1982 by Bodeutsch et al. a percentage IgA containing plasma cells (IgA%) less than 70 was very specific for SS<sup>6</sup>. With the QIH criterion some patients previously not fulfilling ESG criteria for SS because of a focus score < 1 could be identified as having SS. Thus, when shifting towards more objective and target-organ specific signs, examining SGT biopsies immunohistochemically instead of or next to histologically appears favourable. The objective of this study is to compare disease-sensitivity and -specificity of histological (focusscore) results to immunohistological (IgA%) results from

examination of SGT biopsies to evaluate both methods which have been part of routine examination of SS in our department for over 10 years now.

## **METHODS**

From 1988-1998 a total of 279 SGT biopsies were taken of patients with symptoms suggestive for SS. Both LFS and IgA% scores were assessed in the biopsies. Samples were assigned to one of four groups: a group with LFS>1.0 and IgA%<70 (i.e. histological diagnosis 'SS'), a group with LFS<1.0 and IgA%>70 (i.e. histological diagnosis 'no SS'), and two mismatch groups A and B. Group A consisted of samples with LFS>1.0 and IgA%>70 and group B of samples with LFS<1.0 and IgA%<70. The mismatch groups A and B were further classified using the following major and minor objective serological criteria: elevated gammaglobulin level and presence of SS-A- and SS-B-autoantibodies were considered major objective parameters suggesting SS, whereas presence of RF or ANA were considered minor objective parameters suggesting SS. If both major parameters were present, or if one major plus one minor parameter were present, the mismatch patient was considered as having SS. Instead, if patients had only minor criteria, they were considered as having (secondary) sialadenitis but not Sjögren's syndrome. In the double positive or double negative concordant biopsies groups the number of false positive and false negative LFSs and IgA%s was estimated based on the oldest half (first five years) of data (n=149). Since these biopsies showed concordant immunohistological results, not only the serological classification criteria used in the mismatch groups but also the current ESG criteria could be used in these groups, enabling comparison of both definitions.

## **RESULTS**

Of the 279 biopsies 63 showed a LFS >1 and an IgA%<70 which immunohistologically classified these patients as having SS. A total of 186 biopsies showed an LFS <1 and an IgA%>70 which led to the immunohistological diagnosis 'no SS'. Furthermore 16 biopsies (group A) showed a LFS >1 and an IgA%>70, and 14 biopsies (group B) showed a LFS <1 and an IgA%<70. Thus the diagnosis based on the two different histological criteria did not match in 30 of 279 biopsies (11%). Additional serological analysis (using the major and minor objective criteria as described in the Methods section) of these two "mismatch" groups classified 12 out of 16 positive LFS (75%) and 7 out of 14 (50%) positive IgA% scores as not having SS. Vice versa, based on the serological classification criteria, 4 out of 16 (25%) negative IgA% scores and 7 out of 14 negative LFS (50%) could still be classified as having SS. (Tables I,III).

In the further analysed double positive concordant group (n=31) 71% could be classified as having SS when applying the current ESG classification criteria. The remainder 29% strictly spoken does not have SS but has a (immuno-)histological picture which is strongly suggestive for SS. Of this latter group (n=9) 44% has SS-A/SS-B presence and therefore are positive for two objective and most

**TABLE I - Characteristics of patients in mismatch groups**

	Initials	Age	Sex	Ro	La	Gam.Glob	RF	ANA	Comorbidity	LFS	IgA%	SS?
<b>LFS &gt;1 and IgA% &gt;70 (mismatch group A)</b>												
1	G. K.	72	F	-	-	6.2	-	+	RA	1.8	72.6	No
2	R. B.	54	F	-	-	7.5	+	+	RA	2.3	82.8	No
3	V. W.	55	F	-	-	17.1	-	+	UCTD	1.1	84.9	Yes
4	J. K.	55	F	-	-	10.8	-	+	Sicca syndrome	1.2	79.0	No
5	H. P.	72	F	-	-	12.0	+	+	RA	3.2	72.8	No
6	B. T.	62	F	-	-	9.1	-	-	RA	2.1	86.4	No
7	Z.	47	M	-	-	12.2	+	+	RA	1.4	95.7	No
8	D.	72	F	-	-	12.6	+	+	RA	1.7	87.5	No
9	V.	67	F	+	+	12.2	+	+		2.2	72.6	Yes
10	H.	72	F	-	-	19.6	+	+	RA	1.7	77.9	Yes
11	G.	69	M	-	-	12.7	+	+	UCTD	1.2	74.9	No
12	J.	49	M	-	-	13.4	+	-	RA	1.0	84.2	No
13	B. V.	48	F	+	+	18.6	-	+		1.0	72.9	Yes
14	W. I.	50	F	-	-	9.6	-	+	SLE	1.7	81.5	No
15	B.	69	F	-	-	15.1	-	+		4.7	77.0	No
16	W. E.	65	F	-	-	7.5	-	+		1.9	77.5	No
<b>LFS &lt;1 and IgA% &lt;70 (mismatch group B)</b>												
1	C.	78	F	+	+	20.0	+	-	RA	0.9	23.9	Yes
2	D.	31	F	-	+	22.8	+	+	SLE	0.8	58.9	Yes
3	M.	65	F	-	-	12.7	-	+	Raynaud	0.8	69.3	No
4	W. E.	83	F	-	-	11.8	+	-	RA	0.7	27.9	No
5	V. S.	42	F	-	-	9.4	-	-		0	65.5	No
6	G.	50	M	+	+	17.4	+	+		0	66.6	Yes
7	V. O.	45	F	+	+	16.4	-	+	Scleroderma	0	67.5	Yes
8	L.	52	M	-	-	8.3	-	+	Sicca syndrome	0	49.2	No
9	D. I.	50	F	-	-	11.2	+	-	Fibromyalgia	0	69.0	No
10	E.	50	M	+	-	10.0	-	+		0	57.1	Yes
11	B. E.	34	M	-	-	17.2	-	+		0.9	64.7	Yes
12	B. R.	43	F	-	-	8.1	-	+		0.2	60.5	No
13	W.	48	M	-	-	7.4	+	-	RA	0.3	66.2	No
14	S. J.	33	F	+	-	18.2	-	+		0.8	69.4	Yes

M, male; F, female; Ro, La, RF, ANA: +, positive, -, negative; UCTD, undefined connective tissue disease; SLE, systemic lupus erythematoses; SS, Sjögren's syndrome, classified according to serological criteria. Gam.golb, Gammaglobulin (level >15.5 g/l is considered as elevated).

diseasespecific parameters. In the double negative group (n=90), which as a consequence already lacks one of 6 items (no LFS>1), 31% fulfilled 3 out of 6 items (oral and ocular sicca symptoms, ocular sicca signs) of the ESG classification criteria, but only one patient fulfilled 4 out of 6 items, leading to diagnosis SS (Tables II, III).

**TABLE II - Characteristics of patients in concordant groups**

	n	Oral sicca (%)	Ocular sicca (%)	Ocular test (%)	Ro/La (%)	ANA (%)	RF (%)	Gamma (%)	SS (%)	SS (4+) (%)
LFS <1, IgA% >70	90	64	72	56	9	47	30	10	13	1
LFS >1, IgA% <70	31	71	61	65	48	74	71	39	61	71

ANA, the presence of anti-nuclear antibodies; RF, the presence of rheumatoid factor; Gamma, elevated gammaglobulin level (>15.5 g/l); SS, Sjögren's syndrome, classified according serological criteria; SS (4+), diagnosis of Sjögren's syndrome based on four positive items of ESG classification criteria.

Oral sicca: xerostomia symptoms as described in ESG classification criteria; ocular sicca: xerophthalmia symptoms as described in ESG classification criteria; ocular test: Schirmer-I test positive (<5 mm in 5 min).

**TABLE III - Serological classification overview**

	n	SS (serological)	SS (4+)
<b>Concordant biopsies</b>			
LFS <1, IgA% >70	90	13% (12)	1% (1)
LFS >1, IgA% <70	31	61% (19)	71% (22)
<b>Disconcordant biopsies</b>			
LFS >1, IgA% >70	16	25% (4)	n.a.
LFS <1, IgA% <70	14	50% (7)	n.a.

SS, Sjögren's syndrome, classified according serological criteria; SS (4+), diagnosis of Sjögren's syndrome based on four positive items of ESG classification criteria; n.a., not applicable since the disconcordant biopsy itself is part of these criteria.



When applying the serological criteria that were used for classification of the mismatch-groups, in the double positive group (n=31) 61% could be classified as having SS, whilst in the double negative group (n=90) 13 % could be classified as having SS.

## DISCUSSION

In this analysis of collected SGT biopsy data the additional value of immunohistological examination of SGT was confirmed. When both focus-score and IgA% are assessed, an objective, target-organ specific diagnosis could be established in the majority (89%) of cases. However an unambiguous histological diagnosis is not necessarily equivalent to an unambiguous clinical diagnosis, as can be concluded from the additional analysis of concordant biopsies. Although this analysis showed that SGT biopsy contributes considerably in distinguishing between SS and non-SS patients, biopsy results therefore still need further confirmation by e.g. serological parameters, unless a more disease specific and sensitive tissue-marker is available.

In 11% of cases the conclusion of histological examination of SGT biopsies (focus-score) did not match with the immunohistological result (IgA% score). In these so called "mismatch" cases the IgA% appears superior to the focus score when objective, serological findings are taken into account. After this serological classification, both the numbers of false positive and false negative IgA% scores were considerably lower than the numbers of false positive and false negative focus scores.

In our model chosen for the classification of the two mismatch groups serological parameters were used as objective decisive data, since a golden standard to compare the SGT biopsy results with is lacking. Furthermore none of the existing classification-criteria could be used for categorisation of patients within the mismatch groups because the focus-score itself is part of these criteria. Not only should the model contain objective decisive parameters, it should also enable distinguishing (secondary) focal sialadenitis and secondary Sjögren's syndrome. The latter term should be used with caution, i.e. if next to a LFS >1 additional objective signs of Sjögren's syndrome are present in a patient with connective tissue disease. Thus, a patient suffering from rheumatoid arthritis (RA), with a LFS >1, but no other objective signs of Sjögren's syndrome should be classified as having secondary focal sialadenitis, not Sjögren's syndrome.

Previous findings of different immunohistochemic characteristics in SGT support the need to distinguish between sialadenitis and (secondary) Sjögren's syndrome. The combination of a LFS>1, combined with an IgA%>70 and a normal La-expression pattern is typically observed in sialadenitis, as seen in RA patients, whereas in primary SS and secondary SS (e.g. next to RA) a LFS>1, combined with an IgA%<70 and an abnormal SS-B-expression pattern was found, suggesting a different pathogenesis<sup>11</sup>. Furthermore auto-immune disease (such as RA) patients with a LFS>1 (i.e. sialadenitis) but without any signs or symptoms of SS

have been described previously<sup>6, 7</sup>. Therefore in this model differentiation between sialadenitis secondary to RA versus Sjögren's syndrome was enhanced by attributing more value to elevated gammaglobulin-level and presence of SS-A- and SS-B-autoantibodies. These parameters appear to be more Sjögren's syndrome-specific objective parameters than RF and ANA, which are also frequently present in e.g. RA patients. This is further supported by the measured percentages of serological markers present in histological positive patients as compared to histologically negative patients (table II). However, the presence of SS-A- or SS-B-antibodies is almost inevitably associated with positive ANA testing (but not vice versa). Therefore applying more strict disease-specific criteria in the model for categorisation of the mismatch groups was also considered by comparing SGT results to SS-A- or SS-B- antibodies presence only. In Sjögren's syndrome presence of these antibodies is by far the most diseasespecific serological sign to date. When presence of SS-A- or SS-B-autoantibodies was considered as decisive for the diagnosis of Sjögren's syndrome, the results showed the same pattern of false negative and false positive scores (data not shown). The conclusion remained that the IgA% score in these cases is superior over the focus-score. However, the latter model leaves no place for SS-A- or SS-B-autoantibodies seronegative Sjögren's syndrome patients, which are generally believed to form about 20% of the Sjögren's syndrome population<sup>12-15</sup>.

Should the focus score be considered as obsolete? Our data suggest that the combination of both focus-score and IgA% score can have a synergistic value for the accuracy of diagnosis. In cases of doubt, the other parameter can direct towards the right diagnosis. In this study the number of false negative IgA% scores was remarkably low. An IgA% >70 in combination with a focus score >1 should therefore be interpreted as a false positive focus score. The interpretation of a focus score <1 in combination with an IgA% <70 is less clear, since both false negative focus scores and false positive IgA% scores appear to occur about equally in this mismatch group. However, in the majority of cases assessment of both focus-score and IgA% score leads to an unambiguous histological conclusion. Nevertheless whether histological and immunohistological findings match or not, neither of them have been shown to be 100% sensitive and specific, as was also depicted in the analysis of concordant biopsies. Therefore these SGT parameters should still be related to other available objective data such as the serological profile. An additional advantage of immunohistological examination of SGT is that immunohistological changes, such as %IgA containing plasmacells, are not necessarily associated with lymphocytic foci and, in contrast to histological findings, are much more diffusely located throughout the minor salivary glands tissue<sup>6</sup>. As a consequence the sampling error with regard to the %IgA containing plasmacells, or other yet to develop immunohistological disease specific markers, is less than that of the focus score.

In conclusion, immunohistological examination of SGT provides valuable additional information and reduces sampling error thereby increasing accuracy of diagnosis.

Thus, in order to acquire a maximum of relevant information out of the target organs in SS histological examination of SGT only is not sufficient and should be accompanied by immunohistological examination as well. In other words: two sub-optimal tissue-markers in terms of disease specificity and sensitivity together provide more sensitivity and specificity than applying only one of them.

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## CHAPTER 6

# **Reversibility of histological and immunohistological abnormalities in sublabial salivary gland biopsies following treatment with corticosteroids in Sjögren's syndrome**

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## **ABSTRACT**

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by specific lesions in exocrine glands, so sublabial minor salivary gland biopsy (SLGB) plays an important part in its diagnosis. The extent and composition of the lymphocytic infiltrate in SLGB specimens can be considered as target organ specific parameters. They are quantified after histological and immunohistological examination by a focus score (describing the extent of the infiltrate) and IgA% score (describing the composition of the infiltrate), respectively. However, little is known about the factors that contribute to the extent and composition of the infiltrate and whether these features are reversible as repeated SLGBs are rarely performed. A patient with SS is described who underwent SLGBs before and after treatment with high dose corticosteroids. After treatment there was not only clinical improvement, but also improvement in the histological and immunohistological parameters. Although these findings need to be confirmed in further studies, this suggests that histopathological changes may be reversible in SS. Furthermore, it shows that the potential effects of corticosteroid use should be taken into account when interpreting SLGB specimens. When clinical changes do parallel histological changes, repeated SLGBs might offer a marker for disease activity in patients with SS.

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by specific lesions in exocrine glands<sup>1</sup>. Salivary and lachrymal glands are most prominently involved in this condition, leading to organ specific symptoms such as xerostomia and xerophthalmia. Apart from these local symptoms, most patients also suffer from systemic manifestations such as fatigue, arthralgia, and myalgia. Furthermore, patients with primary SS—that is, without comorbidity from other rheumatic diseases— have an increased risk of developing a non- Hodgkin's lymphoma which is 44 times greater than the rate in the general population<sup>2</sup>.

Since the first description of SS by Henrik Sjögren, many criteria have been proposed for the diagnosis or classification of this syndrome<sup>3-6</sup>. Histological examination of sublabial minor salivary gland biopsy (SLGB) specimens is an important factor in these criteria. The extent and composition of the lymphocytic infiltrate in the SLGB specimens can be considered as target organ specific parameters. The number of lymphocytic foci per 4 mm<sup>2</sup> in the SLGB specimen is expressed as a focus score. However, it is well known that the focus score lacks both sufficient sensitivity and specificity for the diagnosis of SS, depending on whether a focus score of >1 (leading to decreased specificity) or >2 (leading to decreased sensitivity) is taken as the criterion for diagnosis<sup>7-9</sup>. In contrast to the focus score, immunohistological examination of the infiltrate has been shown to have both high disease specificity and sensitivity<sup>7,10</sup>. This method is based on quantification of plasma cells expressing different isotypes of immunoglobulins (IgA, IgM, IgG). A percentage of IgA containing plasma cells (%IgA) of less than 70, or of IgM containing plasma cells (%IgM) of more than 10, is highly specific for SS. So far, little is known about the factors that contribute to the lymphocytic infiltrate. Manthorpe *et al* recently reported the possibility that patients might “smoke” their lymphocytic infiltrate away<sup>11</sup>. In their study the focus scores found in SLGB specimens of 355 patients with either SS or stomatitis sicca were compared with the smoking habits of the patients, and a lower focus score was found in patients who smoked. It appears, however, that external factors may influence the lymphocytic infiltrate seen in SLGB specimens. Medication may well be another external factor which influences the lymphocytic infiltrate. These factors should be taken into account when interpreting the SLGB results.

This case report describes a patient with SS in whom changes occurred in the lymphocytic infiltrate in the SLGB specimen after treatment with corticosteroids.

## CASE REPORT

A 35 year old man with Klinefelter's syndrome was referred with signs suggestive of SS. He complained of dry eyes and mouth, both of which had been present for 10 months. Shortly after onset of the dry eyes he had started to use artificial tear droplets. He also suffered from fatigue and had a history of back pain and a painful but not swollen left hand for 10 years.

For the previous 8 months he had also had a painful left knee. He did not smoke or use any medication. Physical examination revealed no abnormalities other than

signs compatible with Klinefelter's syndrome (gynaecomastia and spare body hair pattern), except for increased conjunctival injection. Laboratory findings included erythrocyte sedimentation rate (ESR) of 10 mm/1st h, C reactive protein (CRP) <5 mg/l, normal blood count, normal liver function tests, normal kidney function, and slightly raised gammaglobulin (16.6 g/l) without paraproteins on immunoelectrophoresis. Further testing revealed positive antinuclear antibody staining on indirect immunofluorescence with a homogeneous pattern. Tests for rheumatoid factor were negative. No autoantibodies such as anti-SS-A, anti-SS-B, anti-Sm, anti-dsDNA, and anti-RNP could be detected either by immunoblotting or by counter immunoelectrophoresis. Ophthalmic examination revealed a keratoconjunctivitis sicca with abnormal rose bengal staining of the cornea, diminished tear drop break up time (3 seconds), and Schirmer's test of 0 mm in 5 minutes for both eyes. Histological examination of the four glands from the SLGB showed 1.0 lymphocyte aggregates per 4 mm<sup>2</sup>—that is, a focus score of 1.0. Quantitative immunohistological examination gave %IgA of 64. Based on the European Study Group diagnostic criteria, a diagnosis of SS was established<sup>5</sup>. Local treatment with eye droplets was started to treat the keratoconjunctivitis sicca. After several weeks, however, the patient developed a painless dystonia of the left hand, with the third to fifth finger as well as the wrist in fixed hyperflexion. The left foot showed a slight inversion and internal rotation. Serological testing for anti-dsDNA, antineutrophilic cytoplasmic, and anticardiolipin antibodies and lupus anticoagulants appeared to be negative. Further neurological examination showed no abnormalities by electromyography (EMG) or magnetic resonance imaging (MRI) of the brain. However, single photon emission computed tomography (SPECT) with iodobenzamide (IBZM) labelled with <sup>123</sup>Iodine revealed decreased uptake in the right striatum (striatum:occipital cortex ratio 1.53 on the left, 1.41 on the right, mean normal (2SD) value 1.66 (0.16)), indicating a loss of D2 receptors in the right striatum (neurological details have been published previously)<sup>12</sup>. A diagnosis of neurovasculitis located in the right striatum was made, and was considered to be an extraglandular complication of SS. Treatment with high dose prednisone (60 mg daily) was started and within 2 weeks the dystonia and the sicca symptoms had disappeared. Re-examination of the lachrymal glands showed a normal Schirmer's test (15 mm in 5 minutes for both eyes), normal tear drop break up time, and normal rose bengal staining of the cornea. After 2 months of treatment the medication was tapered to a dose of 30 mg/day. At that time, and after informed consent had been obtained, a second SLGB specimen was taken to examine the histological and immunohistological changes in the lymphocytic infiltrate. This specimen, in contrast to that taken before treatment, showed not only a normal focus score (0.4) but also a normal IgA% score of 80 on examination of the four glands. Thus, in addition to an improvement in the clinical symptoms, the lymphocytic infiltrate appeared to have normalised after corticosteroid treatment. After 1 month, however, there was a relapse in both sicca and hand symptoms on prednisone 30 mg daily. The dose of prednisone was increased to 60 mg daily and



azathioprine was added to enable the corticosteroid dosage to be re-tapered. Again the symptoms disappeared and returned after tapering of the prednisone dose. Azathioprine was therefore stopped and replaced with cyclophosphamide (100 mg daily) which enabled the corticosteroid dose to be successfully tapered.

## DISCUSSION

This case report describes a patient with SS complicated by neurovasculitis, manifested by dystonia of the left hand and foot. After treatment with high doses of corticosteroids, both the sicca symptoms and the dystonia quickly disappeared. Furthermore, at the same time this treatment resulted in normalisation of the histological and immunohistological abnormalities in the SLGB specimen. This suggests a relation between changes in clinical symptoms and histological and immunohistological changes. Repeated SLGBs might therefore offer an objective, target organ specific method for evaluating treatment since both the clinical and (immuno)histological signs appear to be reversible. This is important because there is, as yet, no clear marker of disease activity for SS and evaluation of treatment is primarily based on subjective parameters.

The histological findings of an SLGB specimen already play an important part in several criteria sets for SS. The diagnosis of SS was made because the patient met the following four of the six ESG criteria: the presence of ocular and oral symptoms for more than 3 months, ocular signs (abnormal Schirmer's test, abnormal rose bengal staining), and a focus score of 1.0. In the second SLGB specimen, however, a normal focus score was observed. If this had been the initial SLGB specimen, the diagnosis of SS would have been less likely and could have been missed, particularly since, at that time, the sicca signs had disappeared. Although the normalised second SLGB specimen might be found coincidentally as a result of sampling error, examination of both specimens included four glands and both samples were examined by two independent pathologists. Furthermore, Bodeutsch *et al* showed that the %IgA containing plasma cells are not necessarily associated with lymphocytic foci and, in contrast to the focally situated lymphocyte aggregates, are much more diffusely distributed throughout the tissue of the minor salivary glands<sup>7</sup>. Thus, the sampling error with regard to the %IgA containing plasma cells is much less than that for the focus score.

In our opinion, therefore, normalisation of both the focus score and the IgA% score gives a reliable result. This case shows that the effect of corticosteroids on the extent and composition of the infiltrate in the target organ in patients with SS cannot be excluded. The same phenomenon might appear when other immunosuppressive agents such as disease modifying antirheumatic drugs (DMARDs) are used. To minimise the potential bias of immunosuppressive agents, it is therefore important to take the SLGB specimen before starting anti-inflammatory treatment. As patients with rheumatoid arthritis often also suffer from SS, the possible impact of treatment with DMARDs or corticosteroids on histological and quantitative immunohistological examination of an SLGB specimen

should be taken into consideration in the diagnosis of SS. Since SLGB plays an important part in the diagnosis of SS, further studies need to be performed to establish which factors contribute to the composition of the infiltrate in these exocrine glands. However, the suggested dynamic properties of histopathological changes need to be confirmed in further studies.

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## CHAPTER 7

# Etanercept in the Treatment of Patients with Primary Sjögren's Syndrome: A Pilot Study

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## ABSTRACT

### **Objective.**

This pilot study evaluated the effect of anti-tumor necrosis factor- $\alpha$  antiinflammatory treatment with etanercept (Enbrel®) on sicca, systemic, and histological signs in patients with primary Sjögren's syndrome (SS).

### **Methods.**

Fifteen patients with well defined primary SS were treated with 25 mg etanercept subcutaneously twice per week during 12 weeks, with followup visits at Weeks 18 and 24. Evaluation measures included a Multidimensional Fatigue Inventory (MFI) questionnaire, serological monitoring, salivary flow tests, Schirmer test, rose bengal cornea staining, and tear film breakup time. A sublabial minor salivary gland biopsy was performed at baseline and at Week 12 and lymphocytic focus score and percentage IgA-containing plasma cells (IgA%) were assessed.

### **Results.**

No increase of salivary or lachrymal gland function was observed in any participant. In 4 patients a decrease of fatigue complaints was noted, which was also reflected by decreased scores in the MFI questionnaire. Reduced erythrocyte sedimentation rate was observed in 3 of 4 patients with reduced fatigue. No significant change of lymphocyte focus score or IgA% was observed. Arepeated treatment up to 26 weeks showed the same results.

### **Conclusion.**

A 12-week or prolonged treatment of etanercept 25 mg twice weekly did not appear to reduce sicca symptoms and signs in SS. However, etanercept treatment may be beneficial in a small subgroup of SS patients with severe fatigue. Etanercept 25 mg twice weekly did not affect minor salivary gland biopsy results.

## INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune exocrinopathy of unknown origin. As a consequence of exocrinopathy, patients present with sicca signs such as irritated eyes, a dry mouth, chronic cough, and dyspareunia. Other manifestations of SS include nonspecific systemic symptoms such as moderate to severe invalidating fatigue, arthralgia, myalgia, and intermitting fever<sup>1</sup>. Patients with SS have an increased risk to develop non-Hodgkin's lymphoma, which affects about 5% of these patients<sup>2</sup>.

The diagnosis of SS is frequently guided by classification criteria sets. Within the recently proposed US/European consensus group classification criteria<sup>3</sup>, objective items such as presence of anti-SSA or anti-SSB autoantibodies and minor salivary gland biopsy showing a lymphocytic focus score (LFS) > 1 are emphasized. In addition to the LFS, which describes the extent of a lymphocytic infiltrate, the composition of the infiltrate can also serve as a diagnostic tool. For example, the percentage IgA-containing plasma cells (IgA%) appears to have greater disease specificity and sensitivity than the LFS<sup>4,5</sup>. Apart from the discomfort patients with SS experience from sicca symptoms (e.g., sleep disturbance), the effect of systemic symptoms is often underestimated. Moderate to severe fatigue is a frequent complaint<sup>6</sup> that disables a subgroup of SS patients to such an extent that it leads to incapacity for work and limits social activities, leading to social isolation.

To date, no effective systemic treatment is available for SS. Disease modifying antirheumatic drugs (DMARD) that are successful in the treatment of several other rheumatic diseases did not turn out to be a successful overall approach in SS<sup>7</sup>. Treatment is therefore mainly symptomatic and consists of artificial eyedrops and saliva. Pilocarpine has been shown to enhance autologous production of both saliva and tears in patients with SS<sup>8</sup>. Although some efforts were undertaken to treat fatigue complaints using conventional or experimental drugs, none has been proven to effectively reduce fatigue in patients with SS<sup>9,10</sup>. Thus, while local symptoms like irritated eyes and dry mouth may be alleviated to some extent, there is neither a systemic nor a symptomatic treatment available to reduce moderate to severe incapacitating fatigue in SS.

Recently, biological agents inhibiting the proinflammatory cytokine tumor necrosis factor (TNF- $\alpha$ ) have been approved for the treatment of rheumatoid arthritis (RA) and chronic juvenile arthritis<sup>11,12</sup>. Promising results have also been presented for the efficacy of anti-TNF- $\alpha$  treatment in psoriatic arthritis, ankylosing spondylitis, Crohn's disease, and Wegener's granulomatosis<sup>13-15</sup>.

Since enhanced TNF- $\alpha$  expression has been observed in exocrine glands of patients with SS<sup>16,17</sup>, anti-TNF- $\alpha$  treatment might potentially suppress inflammation in SS with local and systemic effects. Further, although not well documented, RA patients in practice frequently report not only a beneficial effect of anti-TNF- $\alpha$  treatment on joint symptoms but also a marked improvement of perceived condition and energy. A recent pilot study by Steinfeld and co-workers described treatment with infliximab, an intravenously administered monoclonal chimeric anti-TNF- $\alpha$

antibody, evaluated in 16 patients with SS. Both local (reduced sicca signs) and systemic (reduced fatigue) responses were reported<sup>18</sup>.

Etanercept (Enbrel®), a soluble fully human TNF- $\alpha$ -p75-receptor fusion protein, also interferes with the inflammatory process by binding and inactivating the proinflammatory cytokine TNF- $\alpha$ <sup>19</sup>. Etanercept can be administered subcutaneously by the patients themselves. Our pilot study investigated the potential local and systemic therapeutic effects of etanercept in patients with primary SS. From a diagnostic point of view, a secondary aim of the study was to evaluate whether etanercept treatment would influence minor salivary gland (MSG) biopsy scores. This hypothesis was based on our observation of a normalized LFS and IgA% following immunomodulatory treatment with high dose corticosteroids in a patient with SS<sup>20</sup>.

## MATERIALS AND METHODS

### *Patient selection*

A total of 15 patients with primary SS fulfilling the US/European consensus group classification criteria<sup>3</sup> were included upon giving informed consent. Evidence of sublabial MSG involvement was present in all 15 patients, indicated by a LFS > 1. An IgA-containing plasma cells percentage (IgA%) < 70, shown to be strongly associated with SS<sup>4</sup>, was present in all patients. Patients did not use immunosuppressive agents such as corticosteroids or DMARD.

*Table 1.* Baseline characteristics of patients with primary SS (n = 15). Values are the number (%) of patients, except where noted.

Male: Female	1:14
Mean age, yrs (range)	47.9 (21–80)
Mean disease duration, yrs (range)	3.6 (1–10)
Oral symptoms	15 (100)
Ocular symptoms	14 (93)
Other sicca symptoms	15 (100)
Oral signs	15 (100)
Ocular signs	13 (87)
Positive MSG biopsy	15 (100)
Anti-SSA positive	14 (93)
Anti-SSB positive	13 (87)
IgM-RF positive	14 (93)
Hypergammaglobulinemia	11 (73)
Fatigue	15 (100)
Recurrent fever episodes	7 (47)
Arthralgia	12 (80)
Myalgia	8 (53)

MSG: minor salivary glands, at least one positive biopsy (LFS > 1.0 and IgA % < 70). RF: rheumatoid factor.



Pilocarpine, which potentially increases salivary flow and tear production, was taken in a constant dosage throughout the study by one of the 15 participants. At baseline, patients had to have experience of moderate to severe fatigue, which was the main outcome measure in this study. Table 1 shows the main baseline characteristics of all patients.

### ***Intervention with etanercept***

Patients were treated with etanercept in subcutaneous doses of 25 mg twice per week during 12 weeks of treatment. Followup visits were at 4, 8, and 12 weeks after the start of treatment, followed by post-treatment followup visits at Weeks 18 and 24.

*Measurement of fatigue.* Fatigue was quantified throughout the study using the Multidimensional Fatigue Inventory (MFI) questionnaire. The MFI has been validated for measuring fatigue in Dutch patients with SS6. At each visit the patients completed the MFI questionnaire and a visual analog scale (VAS) scale for perceived disease activity. The MFI consists of 20 questions in 5 scales revealing different dimensions of fatigue (i.e., general fatigue, physical fatigue, mental fatigue, reduced motivation, and reduced activity). Each scale score ranges from 4 to 20, a higher score indicating more severe fatigue<sup>21</sup>. A clinical response regarding perceived fatigue was predefined as a decrease of at least 6 points within a MFI scale [a decrease of 1 point on each item (range 1–5) would decrease a MFI scale score by 4 points].

### ***Evaluation of salivary and lachrymal gland function***

At baseline and at Weeks 4, 12, and 24 unstimulated and stimulated (2% citric acid) combined sublingual and submandibular (SL/SM) salivary flow was measured as recommended by Kalk, *et al*<sup>22</sup>. Unstimulated SL/SM flow and stimulated SL/SM flow was measured for 5 and 10 min, respectively, by manually aspirating saliva with a Monoject syringe. Parotid flow was blocked using Dry-tips™ absorption shields covering the opening of Stensen's ducts on either side. The weight of both the unstimulated and stimulated saliva collecting tubes was electronically measured before and after collection of saliva, including a third control tube that was not used for saliva collection. The latter enabled correction in case of changed circumstances (such as room temperature). At the same time points lachrymal gland function was evaluated using the Schirmer-1 test (i.e., without anesthetic droplets and with closed eyes for a 5 min period). Complete ophthalmologic examination including rose bengal cornea staining and tear film breakup time was performed at screening and at Week 12 by an ophthalmologist.

### ***Serological and histological evaluation***

Serological tests, apart from standard safety tests and gammaglobulin level measured at each visit, included anti-dsDNA and IgM rheumatoid factor measured at screening and at Week 12. All serological variables were assessed using routine standardized measurement procedures. At baseline and at Week 12 patients underwent a sublabial MSG biopsy performed by an experienced oral and cranio-

maxillofacial surgeon. The first biopsy was taken on the left and the second biopsy on the right side of the lower lip. Focus scores were assessed by an experienced pathologist, and computer-aided microscopy was used to quantify the IgA%.

### ***Statistical analysis.***

An intention-to-treat (ITT) analysis was performed comparing scores at baseline and at Week 12. Because MFI scores and VAS scores (Table 2) decreased very rapidly after cessation of etanercept treatment these scores were compared during treatment (Week 8) versus baseline scores. Data were analyzed by a paired t test using the SPSS 9.0 software package.

The pretreatment and post-treatment MSG biopsies (Tables 3 and 4) of the 10 subjects who underwent both biopsies were also compared using a paired t test. In this per-protocol statistical analysis one subject was excluded because her MSG biopsies were assigned the score of 12. Since this is an arbitrarily defined value describing a large confluent lymphocytic infiltrate that does not enable measurement of separate lymphocytic aggregates per area, and this value is not part of a continuous LFS scale, this score was excluded from group comparison. The post-treatment MSG biopsy of this subject, however, was again assigned a LFS of 12 by the pathologist, i.e., no change of the histological picture. Salivary and lachrymal gland function (Table 5) was not tested statistically because of extreme low values in a small study population and no clinical response.

## **RESULTS**

Etanercept was well tolerated in all participating patients with SS. One patient, however, had to temporarily interrupt etanercept treatment at Week 7 because of a prolonged episode of parotitis, which quickly resolved with antibiotics after cessation of etanercept treatment. Due to this interruption, for this patient the data of subsequent scheduled visits were not acquired at the intended time points. Also, the parotitis episode considerably biased erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) values. For this patient it was therefore decided not to include the data acquired after the treatment interruption in the ITT analysis. Three other patients ended their participation before completing the protocol due to lack of efficacy of treatment and were therefore considered as dropouts. Therefore in the ITT statistical analysis the last observation of these 4 patients was carried forward to Week 12.

### ***Systemic signs/symptoms***

A persistent decrease of fatigue was reported by 4 of 15 patients, including the patient who had to temporarily interrupt anti-TNF- $\alpha$  treatment due to parotitis. This was also reflected in the fatigue scores of the MFI and the VAS scores (Table 2, data of responders described separately at the bottom of the table). For the group as a whole a statistically significant decrease of the general fatigue scale within the MFI ( $p = 0.018$ ) as well as the VAS score for perceived disease activity ( $p = 0.045$ )

was observed. In the clinically responding patients the mean score on the general fatigue scale diminished from 16.8 to 8.0, and the score on the physical fatigue scale from 16.2 to 10.0, with the maximum effect reported at Week 8. The effect started after 2–4 weeks of treatment, and disappeared in 3 patients within 2 weeks after cessation of etanercept administration.

*Table 2.* Multidimensional Fatigue Inventory (MFI) scores. Values are mean (SD).

MFI scales	Baseline	Week 4	Week 8	Week 12	Week 18	Week 24
Total, n*	15	15	14	12	11	11
General fatigue	16.3 (3.6)	13.9 (3.4)	12.4 (4.7) <sup>†</sup>	13.1 (3.9)	13.5 (3.7)	13.6 (6.0)
Physical fatigue	15.2 (3.7)	13.2 (3.8)	12.1 (3.2)	13.0 (3.8)	12.5 (4.1)	13.0 (5.5)
Reduced activity	13.0 (3.3)	12.9 (4.4)	12.1 (4.1)	10.3 (3.1)	12.1 (2.9)	12.1 (5.2)
Reduced motivation	11.8 (3.9)	11.1 (3.4)	10.3 (3.2)	10.1 (3.0)	10.5 (3.5)	10.9 (4.7)
Mental fatigue	12.8 (4.4)	11.9 (3.7)	11.3 (4.7)	12.0 (4.5)	10.7 (4.4)	10.3 (4.7)
VAS, mm	70 (19)	61 (21)	50 (16) <sup>†</sup>	59 (17)	60 (13)	66 (20)
Responders only						
General fatigue	16.8 (3.0)	10.3 (1.5)	8.0 (2.0)	10.0 (3.5)	11.0 (3.6)	14.7 (9.2)
Physical fatigue	16.2 (3.6)	10.0 (2.0)	10.0 (2.6)	10.0 (1.7)	10.0 (4.0)	15.0 (8.7)
Reduced activity	12.8 (3.3)	8.7 (0.6)	11.3 (4.2)	8.0 (1.7)	11.0 (3.6)	13.7 (7.6)
Reduced motivation	10.2 (2.3)	7.0 (2.9)	7.7 (3.5)	8.0 (2.6)	7.3 (3.1)	10.0 (5.6)
Mental fatigue	15.3 (4.4)	13.7 (5.5)	11.7 (4.0)	14.3 (4.6)	11.0 (5.2)	12.3 (4.5)
VAS, mm	73 (16)	41 (20)	34 (10)	53 (22)	67 (14)	67 (32)

\* Data of all patients, including the responders. <sup>†</sup>  $p < 0.05$  vs baseline by paired  $t$  test. VAS: Visual analog scale for perceived disease activity.

*Table 3.* Serological and histological data. Values are mean (SD).

	Baseline	Week 4	Week 8	Week 12	Week 18	Week 24
Total, n	15	15	14	12	11	11
ESR, mm	24 (25.6)	24 (26.7)	18 (15.6)	13 (95)	18 (10.5)	20 (11.9)
CRP, mg/l	4.4 (3.8)	4.5 (4.4)	2.8 (1.8)	2.3 (1.6) <sup>†</sup>	3.2 (1.6)	3.4 (3.3)
Gammaglobulin, g/l	16 (7.1)	17 (8.0)	15 (4.2)	15 (4.8)	15 (4.6)	16 (4.3)
LFS	2.22 (1.5)			1.49 (1.0)		
IgA%	56.0 (9.3)			54.3 (8.3)		
IgM RF, U/l	85 (95)			80 (102)		
Responders only						
ESR, mm	19 (18.2)	15 (11.7)	14 (11.9)	12 (12.3)	16 (17.0)	15 (12.2)
CRP, mg/l	3.7 (2.3)	1.7 (0.6)	2.0 (1.0)	2.0 (1.0)	4.0 (2.8)	2.7 (2.1)
Gammaglobulin, g/l	16 (5.4)	16 (5.4)	15 (6.0)	16 (4.0)	15 (5.9)	18 (0.3)
LFS	2.62 (2.0)			2.08 (0.5)		
IgA%	56.1 (16.3)			54.7 (15.6)		
IgM RF, U/l	63 (17)			49 (23)		

<sup>†</sup>  $p < 0.05$  versus baseline by paired  $t$  test. ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, LFS: lymphocytic focus score, IgA%: percentage IgA-containing plasma cells, IgM RF: IgM rheumatoid factor.

In one patient the effect was prolonged, and disappeared after 8 weeks. In 3 of the 4 responders ESR levels and the IgM rheumatoid factor level decreased during etanercept treatment. For the entire group the mean ESR decreased from 24 to 13 mm/h ( $p = 0.058$ ), while the mean CRP values decreased from 4.4 to 2.3 mg/l ( $p = 0.048$ ), although it should be noted that CRP values in general were already low at baseline. Gammaglobulin concentrations however were stable throughout the study in all participants (Table 3). It is noteworthy that one patient appeared to undergo a “reverse” response: she felt worse during anti-TNF- $\alpha$  treatment and much better in the post-treatment followup phase without treatment. All 15 participants remained negative for anti-dsDNA antibody throughout the study.

### ***Sicca signs/symptoms***

An objective substantial improvement of salivary gland function and improvement of lachrymal gland function could not be established in any of the 15 patients. Both Schirmer-1 tests and SL/SM salivary flow measurements showed sustained low to extremely low scores throughout the study (Table 5). Also, no changes were observed in tear film breakup time or rose bengal staining (data not shown).

Subjective improvement of either irritation of eyes or dry mouth was also not reported in the majority of patients. One patient reported less vaginal dryness, leading to resolved dyspareunia complaints, and one patient reported less respiratory tract dryness. Two patients who frequently experienced severe blepharitis reported considerable alleviation of eye symptoms during etanercept treatment. However, once etanercept treatment was stopped, these patients again encountered ocular discomfort due to blepharitis.

*Table 5. Salivary and lachrymal gland function tests. Values are mean (SD).*

	Baseline	Week 4	Week 12	Week 24
Total, n	15	15	12	11
Schirmer-I, mm/5 min	5.0 (5.8)	5.9 (6.2)	5.8 (7.0)	5.6 (9.8)
U-SL/SM flow, ml/5 min	0.1	< 0.01	< 0.01	< 0.01
S-SL/SM flow, ml/10 min	0.4	0.3	0.4	< 0.01
Responders only				
Schirmer-I, mm/5 min	0.3 (0.6)	1.7 (0.6)	2.0 (1.7)	1.3 (1.5)
U-SL/SM flow, ml/5 min	< 0.01	< 0.01	< 0.01	< 0.01
S-SL/SM flow, ml/10 min	< 0.01	< 0.01	< 0.01	< 0.01

SL: sublingual, SM: submandibular, U: unstimulated, S: stimulated with 2% citric acid once per 2 minutes.

### ***Pre- and post-treatment salivary gland biopsy scores***

The (immuno)histological pattern of the pre- versus post-treatment sublabial MSG biopsies interestingly showed a trend to decreased lymphocytic focus scores following etanercept treatment. Only one of 10 samples showed a slightly (0.25) increased post-treatment LFS, while in 7 of 10 samples a decrease varying from

0.2 to 2.4 points was observed in the post-treatment LFS (Table 4). However, the difference in post-treatment LFS ( $p = 0.101$ ) and IgA% ( $p = 0.621$ ) from baseline values was not statistically significant (Table 3).

Thus, the (immuno)histological pattern was compatible with the clinical findings for sicca signs and symptoms. As well no noteworthy differences were observed in sublabial MSG biopsy scores from patients with decreased fatigue versus nonresponding patients. Table 4 shows, surprisingly, that in 3 of 15 subjects a LFS  $< 1.0$  was found at baseline. In all 3 patients a previous diagnostic MSG biopsy had shown a LFS  $> 1.0$ . Furthermore, in 2 of these 3 patients the IgA% at baseline was below 70, which has been shown to be a more disease-specific marker<sup>4,5</sup>. In the third patient a LFS could not reliably be assessed and IgA% could not be assessed at all due to a bad sample. The double MSG biopsies were well tolerated in this study population. One patient complained about postbiopsy numbness of the lip, while all other biopsies were taken without complications. Although in 9 of the 15 participants a previous diagnostic lower lip biopsy had preceded the 2 biopsies taken in this study, no difficulties were encountered assessing LFS and IgA% in the study samples.

*Table 4. Pre- versus post-treatment minor salivary gland (MSG) biopsy scores. (Only the data of double MSG biopsies are shown).*

Patient	Time 0		Time 1	
	LFS	IgA%	LFS	IgA%
1	0.18	60.7	0.22	65.2
2	12	37.1	12	34.4
3	0.5	NA	0.34	62.7
4	3.39	61.0	2.85	54.9
5	4.9	58.2	2.47	65.7
6	1.91	58.4	1.73	59.3
7	2.68	64.0	2.35	48.4
8	1.44	38.8	1.69	43.6
9	0.62	62.5	0.15	49.0
10	2.11	44.2	1.61	48.6

Time 0: pretreatment biopsy, Time 1: post-treatment biopsy, LFS: lymphocytic focus score, IgA%: percentage IgA-containing plasma cells, NA: data not available (unreliable small tissue sample).

### **Extension phase**

After completing the study protocol including the 12 week post-treatment followup without etanercept, all 4 responding patients resumed etanercept treatment 25 mg twice a week. Again, fatigue symptoms decreased quickly (within 2–4 weeks), but as well, after prolonged treatment for up to 26 weeks, no effects on sicca signs or symptoms were noted. No adverse events occurred in patients who participated in this extension phase.

## DISCUSSION

In this pilot study, subcutaneous administration of etanercept 25 mg twice weekly in patients with SS did not improve salivary or lachrymal gland function. However, in a small subgroup of patients, there was markedly reduced fatigue as well as reduced ESR level, which may indicate a possible systemic response. This study describes 4 of 15 patients who reported less fatigue during etanercept treatment. Although fatigue cannot be assessed objectively, and the numbers of participants in this pilot study were small, their reports were very consistent throughout and following the study. Fatigue scores worsened again in the post-treatment followup phase of the protocol, but after restarting etanercept treatment in the extension phase these patients again reported a quick decrease of perceived fatigue. In these patients the (immuno)histological pattern in the MSG biopsies did not differ from that of nonresponders in terms of fatigue. Indeed, although decreased LFS scores were observed, etanercept treatment did not lead to statistically significant changes in MSG biopsy scores in any patient in this study. In general, etanercept was well tolerated and appeared to be a safe drug for patients with SS. These results contrast in part to observations of Steinfeld, *et al*, who reported a beneficial effect of anti-TNF- $\alpha$  treatment with infliximab on both fatigue complaints and sicca signs in a very similar open label pilot study<sup>18</sup>. This might be explained by differences in patient selection and chosen method of saliva collection, or result from different efficacy of infliximab versus etanercept in SS.

Although both anti-TNF- $\alpha$  agents have potent antiinflammatory effects in RA<sup>11,23</sup>, some recent clinical studies in other autoimmune diseases indeed suggest different clinical efficacy of etanercept compared to infliximab. For example, the differing results of initial studies with infliximab and etanercept in Crohn's disease<sup>15,24,25</sup> are quite similar to our results compared to those of Steinfeld, *et al* in SS. One hypothesis drawn from an *in vitro* study is that while both antagonists block circulating TNF- $\alpha$  molecules, the blocking of membrane-bound TNF- $\alpha$  lasts longer in infliximab treatment compared to etanercept treatment, in which the receptor fusion protein appears to dissociate from the membrane-bound TNF- $\alpha$  some time after having bound to it<sup>26</sup>. As a consequence, a higher etanercept dosing regimen, or shorter dosing interval, might be needed to achieve comparable efficacy to infliximab in SS. Although the exact characterization of systemic signs and symptoms in patients with SS is unknown, it is noteworthy in this respect that a subgroup of patients reported less fatigue during etanercept treatment yet none of these patients showed functional improvement of the glands. However, since measuring serum concentrations of TNF- $\alpha$  was not part of the study design, the hypothesis that circulating TNF- $\alpha$  levels might be associated to fatigue cannot be verified in this study. For efficacy of anti-TNF- $\alpha$  treatment in SS, careful patient selection may be important as well, since reversibility of decreased glandular function in an advanced stage of the disease is questionable. To compensate for this issue in future studies, one might define a minimum glandular rest function at baseline or challenge patients with pilocarpine to test for potential reversibility of

glandular function. This might increase the probability of successful etanercept intervention resulting in a functional response of the glands. Measuring parotid flow next to sublingual and submandibular flow might have given additional information about the functional status of salivary glands. However, from the evidence that the majority of patients did not report improvement of dry mouth, and macroscopic inspection of the absorption shields that were used to block parotid flow into the oral cavity did not show improvement as well, it is unlikely that a clinically important improvement of salivary flow was overlooked by not measuring parotid flow.

In conclusion, etanercept administered subcutaneously in a conventional RA dosing regimen of 25 mg twice weekly did not lead to improvement of sicca signs and symptoms in 15 patients with primary SS. In a small subgroup of patients, however, a presumed systemic response was observed, indicated by a reduction of moderate to severe fatigue complaints in these patients. Since this etanercept dosing regimen did not result in statistically significant histological changes in sublabial MSG gland biopsies, there are no indications that diagnostic biopsies taken, for example, in patients with RA are biased by concomitant etanercept treatment. Additional studies might elucidate the possible fatiguereducing mechanism of anti-TNF- $\alpha$  treatment. The efficacy of etanercept treatment in reducing oral and ocular dryness in SS should be evaluated in studies with a higher dosage or shorter dosing intervals.

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## **CHAPTER 8**

# **Summary**

Sjögren's syndrome (SS) is considered a chronic autoimmune exocrinopathy, predominantly involving salivary and lacrimal glands. Exocrine glands that are located in the upper airways, the vagina and possibly the skin may also be affected. As a result of exocrinopathy the clinical manifestations of SS typically include sicca symptoms such as dry eyes and dry mouth. In addition the syndrome is clinically characterised by non-specific flu-like symptoms such as fever, arthralgia, myalgia and fatigue. SS is thought to affect approximately 0.5-1.0% of the population in the US and Europe, and has been subject of extensive scientific research. One of the continuing dilemmas in both research and daily clinical practice however is how to define SS. As a consequence, data obtained can often not be easily compared. Importantly, in daily clinical practice some physicians diagnose SS by using classification criteria sets, whereas others prefer to establish a diagnosis based on the clinical symptoms and signs that constitute the syndrome originally described by Henrik Sjögren, a Swedish ophthalmologist.

A general introduction to this thesis is provided in **Chapter 1**. In this chapter a historical overview of the evolving concept of SS, starting from the early descriptions of the syndrome by Henrik Sjögren and others, followed by a description of various proposed classification criteria and consecutive modifications that have been applied to them, is given. Difficulties that are currently encountered in the development of an objective diagnostic model and disease activity measurement tool for SS are discussed here and provide the background for this thesis.

In the current 2002 American/European Consensus Group (AECG) classification criteria for SS objective items, such as serum presence of anti-SS-A and anti-SS-B antibodies (anti-SS-A, anti-SS-B), are emphasized. In the sera of approximately 20% of SS patients no anti-SS-A or anti-SS-B can be detected at the time of diagnosis. However, in these 'seronegative' patients anti-SS-A or anti-SS-B may nevertheless be present in other body compartments, e.g. within the exocrine glands or their excreta such as tear fluid or saliva. **Chapter 2** describes a study that is focussed on the relatively understudied group of anti-SS-A and anti-SS-B seronegative SS patients. The aims of this study were to assess whether anti-SS-A and anti-SS-B could be detected in tear fluid samples of our seronegative SS patient population, and to examine whether seroconversion to a positive profile occurred after disease had progressed for at least five years since diagnosis. If either of these was the case, the "intrinsic" disease-sensitivity of anti-SS-A and anti-SS-B could be higher than previously assumed. The results of this study show that there are no indications for seroconversion over time from an anti-SS-A and anti-SS-B negative to positive serological profile in patients previously tested seronegative. However, in some of these seronegative patients anti-SS-A presence can be found in tear fluid samples. In seropositive patients that served as controls both anti-SS-A and anti-SS-B presence was demonstrated in tear fluid samples. A remarkably low amount of tear fluid (approximately 5 µl) appeared to be enough to detect anti-SS-A by ELISA. However, the number of examined seronegative tear fluid samples was small, and measured

sensitivity was low. Thus, tear fluid analysis for presence of anti-SS-A or anti-SS-B in seronegative SS patients has yet to prove its additional diagnostic value.

Implications from this study in clinical practice are:

1. Repeated serological analysis for anti-SS-A or anti-SS-B presence in previously known seronegative patients is of no additional diagnostic value.
2. Although optimised tear fluid analysis might reveal anti-SS-A or anti-SS-B presence in seronegative SS patients, thereby facilitating the diagnosis of Sjögren's syndrome, the yield of tear fluid analysis in these patients is currently too low to be advocated in clinical practice

The disease sensitivity of anti-SS-A and anti-SS-B has been reported to be 60-75 % and 30-50 % respectively, whereas disease specificity of particularly anti-SS-B is generally considered as reasonably high. Anti-SS-B is mostly found in SS and SLE patients, and rarely present in other diseases or normal healthy subjects (NHS). However, as mentioned above, approximately 20% of SS patients lack serum presence of anti-SS-A and anti-SS-B. Because of this limited disease sensitivity and specificity, the search for an autoantibody showing a better disease sensitivity and specificity profile continues. Anti-alpha-fodrin autoantibodies (anti-alpha-fodrin) are amongst the proposed candidate serological markers for SS. Previously the disease sensitivity and specificity profile of anti-alpha-fodrin has been claimed to be superior to that of anti-SS-A and anti-SS-B. From initial studies a disease sensitivity of 96% was reported for anti-alpha-fodrin in primary SS, whilst disease specificity of these antibodies was comparable to that of anti-SS-B. However, the data presented in **Chapter 3** show that the previously reported superior disease sensitivity and specificity profile of anti-alpha-fodrin could not be reproduced in our cohort of well-defined SS patients.

This was concluded based on multiple ELISA measurements that were combined with at least one alternative biochemical technique for confirmation of obtained data. In contrast to anti-alpha-fodrin, all anti-SS-A and anti-SS-B activities detected in our sera by ELISA could be confirmed by using alternative biochemical techniques. Also, in this study the observed sensitivity of anti-SS-A and anti-SS-B was higher than that of anti-alpha-fodrin. In addition, a considerable overlap between the presence of anti-SS-A, anti-SS-B and anti-alpha-fodrin was observed. A potential contributing role for anti-alpha-fodrin measurement to detect SS patients that are negative for anti-SS-A and anti-SS-B appears therefore unlikely. In addition, although this study was not designed to evaluate the disease specificity of anti-alpha-fodrin, it is unlikely to exceed that of anti-SS-B considering the detection of some anti-alpha-fodrin positive sera in rheumatoid arthritis (RA) patients.

Therefore, in clinical practice, the measurement of anti-alpha-fodrin autoantibodies does not add much to the diagnosis of Sjögren's syndrome.

In view of the current concept of Sjögren's syndrome (SS) as an autoimmune exocrinopathy of unknown origin, xerosis (dry skin), which is frequently reported in SS, has been assumed to result from cutaneous exocrinopathy. However, convincing histological evidence for this hypothesis is lacking. Theoretically, if the typical infiltrating lymphocyte aggregates, such as seen in biopsy specimens of salivary glands, could also be found in skin tissue, a diagnostic skin biopsy could serve as an alternative to salivary gland tissue biopsy in SS patients. Exocrine glands in the human skin include sebaceous glands as well as eccrine or merocrine (formerly known as apocrine) sweat glands. The sebum, rather than sweat, controls moisture loss from the epidermis. Decreased sebaceous gland function is therefore reflected in xerosis. Interestingly all exocrine glands in human skin are under the control of sex hormones. Although SS predominantly affects women, no direct estrogen mediated pathophysiological mechanisms have been identified in previous studies. In **Chapter 4** skin biopsy specimens of SS patients are explored for signs of exocrinopathy. The estrogen-alpha (ER $\alpha$ ) and estrogen-beta (ER $\beta$ ) receptor expression in skin and salivary gland biopsies of SS patients compared to NHS, are also described in this chapter. Evidence for inflammatory cutaneous exocrinopathy in SS was not found.

Thus, in clinical practice, a skin biopsy –as an alternative for salivary gland biopsy- is of no use in the diagnosis of Sjögren's syndrome.

In SS patients ER $\beta$  is the predominant ER in skin and salivary gland tissue. This is in line with the previously described predominance of ER $\beta$  in skin and salivary glands of NHS. The observed differentiated ER $\beta$  expression and the presence of ER $\beta$  positive lymphocytes within salivary gland infiltrates in SS patients may reflect a role for ER $\beta$  in the pathogenesis or maintenance of SS. However, further studies should point out whether these observations are of pathophysiological relevance. If so, new pathophysiological and therapeutic concepts for SS may be developed.

Not only the presence of anti-SS-A and anti-SS-B but also a positive salivary gland biopsy is an objective item emphasised in the AECG criteria. A salivary gland biopsy is currently considered positive when the lymphocyte focus score (LFS) is greater than, or equals 1. The LFS represents the number of mononuclear cell infiltrates containing at least 50 inflammatory cells per 4 mm<sup>2</sup>. However, the limited disease sensitivity and specificity of the LFS is well known. Therefore, additional examination of salivary gland tissue might help to differentiate between true- and false positive and negative LFS. One of the methods to do so is quantitative immunohistological (QIH) examination of the salivary gland biopsy. Here the composition of the infiltrate is described in terms of immunoglobulin subtypes (IgG, IgM, and IgA) containing cells. In the QIH criterion introduced in 1982 by Bodeutsch et al. a percentage IgA containing plasma cells (IgA%) less than 70 was very specific for SS. With the QIH criterion some patients previously not fulfilling the classification

criteria for SS because of a LFS < 1 could be identified as having SS. Furthermore, auto-immune disease (such as RA) patients with a LFS>1 (i.e. sialadenitis) but without any signs or symptoms of SS have been described previously, and often fail to meet the QIH criterion. Thus, examining salivary gland tissue biopsies immunohistochemically instead of or next to histologically appears favourable.

In **Chapter 5** the disease-sensitivity and –specificity of histological (LFS) versus immunohistological (IgA%) results of salivary gland biopsy are evaluated. In the majority of cases assessment of both focus-score and IgA% score leads to an unambiguous histological conclusion. However, whether histological and immunohistological findings match or not, neither of them have been shown to be 100% sensitive and specific, as was also depicted in an analysis of concordant biopsies. In 11% of cases the conclusion of histological examination of salivary gland biopsies (LFS) did not match with the immunohistological result (IgA%). In these so called “mismatch” cases the IgA% appears superior to the LFS when objective, serological findings are taken into account. Differentiation between sialadenitis secondary to RA versus Sjögren’s syndrome was enhanced by attributing more value to elevated gammaglobulin-level and presence of anti-SS-A and anti-SS-B. These parameters appear to be more Sjögren’s syndrome-specific objective parameters than rheumatoid factor and ANA, which are also frequently present in e.g. RA patients.

Our data suggest that in clinical practice the accuracy of diagnosis can be enhanced if both LFS and IgA% score of salivary gland biopsy are taken into account. In cases of doubt, the other parameter can direct towards the right diagnosis. In this study the number of false negative IgA% scores was remarkably low. An IgA% >70 combined with a LFS >1 should therefore be interpreted as a false positive LFS. The interpretation of a LFS <1 in combination with an IgA% <70 is less clear, since both false negative LFS and false positive IgA% scores occur with equal frequencies.

Although no effective systemic treatment for SS is available to date it should be noted that in clinical trials treatment efficacy is mainly clinically evaluated using a wide range of non-uniform objective and subjective parameters. A need for an objective disease activity parameter is widely felt.

*Reversibility* is an important component of the currently proposed disease activity model. However, well-documented clinical or histological reversibility has not been reported in SS studies. In **Chapter 6** the results of (immuno)histological examination of salivary gland tissue before and after treatment with high dose corticosteroids in a SS patient are described. Both LFS and IgA% were suggestive for SS before treatment, and normalised after treatment. This case suggests that (immuno)histological changes in SS might be reversible. Thus, repeated salivary gland biopsy may offer an objective tool in evaluating efficacy in therapeutic trials.

Although this observation comes from a single case report, in clinical practice it should be taken into account that false-negative (immuno)histological results from salivary gland biopsies can occur in case of concomitant corticosteroids medication.

In order to further explore the potential reversibility of immunohistological changes in salivary gland biopsies, and to evaluate clinical efficacy, a pilot study in which SS patients were treated with etanercept (Enbrel®), was performed (**Chapter 7**). Etanercept (Enbrel®), a soluble fully human TNF- $\alpha$ -p75-receptor fusion protein, interferes with the inflammatory process by binding and inactivating the pro-inflammatory cytokine TNF- $\alpha$ . Since enhanced TNF- $\alpha$  expression has been observed in exocrine glands of patients with SS, anti-TNF- $\alpha$  treatment might potentially suppress inflammation in SS with local and systemic effects. However, from the data obtained from this pilot study it was concluded that etanercept, administered subcutaneously in a conventional RA dosing regimen of 25 mg twice weekly, did not lead to improvement of sicca signs and symptoms in 15 SS patients. Furthermore, this etanercept dosing regimen did not result in statistically significant histological changes in salivary gland biopsies. Thus, in contrast to corticosteroids treatment, there are no indications that diagnostic salivary gland biopsies results are biased by concomitant etanercept treatment.

In clinical practice, etanercept in a dosing regimen of 25 mg twice weekly, does not offer an effective treatment modality for SS. From a diagnostic point-of-view, it appears that salivary gland biopsies results are not biased by concomitant etanercept treatment.

### **Concluding remarks**

The various components of this thesis can all be considered in the context of the evolving concept of SS in which the focus is currently on improvement of the existing classification criteria and the development of a disease activity measurement tool. In the most recent modification of SS classification criteria, the 2002 AECG criteria, objective signs, i.e. the presence of anti-SS-A or anti-SS-B and a positive salivary gland biopsy, are emphasised. However, both objective signs are limited in disease specificity as well as disease sensitivity. Thus, the further development of classification criteria showing a better performance on disease specificity and sensitivity is still much needed. In this thesis both the serological and the histological item within the current classification criteria were subject of further evaluation. Anti-alpha fodrin antibodies could not be shown to be superior to anti-SS-A and anti-SS-B, and should therefore not substitute anti-SS-A and anti-SS-B presence in the current criteria. Meanwhile the disease sensitivity and specificity of anti-SS-A and anti-SS-B remains limited. Approximately 20 percent of SS patients lack serum presence of these antibodies and continue to do so as disease progresses. Although these antibodies



can also be detected in the saliva and tear fluid of SS patients, it is questionable whether analysis of these exocrine glands excreta will considerably improve the disease sensitivity of these antibodies. A positive salivary gland biopsy is currently defined by a minimum LFS of 1. However, numerous previous studies have shown that both disease specificity as well as disease sensitivity performance of the LFS as histological parameter is poor. One way of improving the disease specificity of salivary gland biopsy may be found in analysis of the composition of the salivary gland infiltrates, e.g. by measurement of the percentage IgA containing plasmacells. The combination of IgA% and LFS was shown to increase the accuracy of diagnosis in SS.

A disease activity model for SS is currently under development. *Reversibility* is an important feature of the proposed model. However, reversibility has not often been objectively documented in SS. Considering the mild, slowly progressing course of disease, and the low rate of patient-reported flares, the actual presence of measurable disease activity in SS is questionable. This may be due to the fact that when SS becomes clinical manifest and diagnosed, patients are apparently in a late stage of disease, with little opportunities for reversibility of already present exocrinopathy. Defining a disease severity (or *damage*) index to start with may be more suitable given the stable clinical course observed in SS. However, so far only a single study of end-organ damage has been performed in SS patients.

In order to diagnose the syndrome before irreversible glandular damage occurs, a screening test for SS will have to be considered in a “pre-sicca” stadium in which the patient may only present with non-specific symptoms. This demands a quick, cheap and preferably non-invasive screening test to rule out or confirm SS in potentially large numbers of patients. However, the need for early diagnosis before irreversible damage has taken place, may not be widely felt before a curative treatment, that enables intervention in the course of Sjögren’s syndrome, is found.



## CHAPTER 9

# Samenvatting

Het syndroom van Sjögren (SS) wordt beschouwd als een chronische auto-immuun aandoening van exocriene klieren, zich met name manifesterend in speeksel- en traanklieren. Tevens kunnen exocriene klieren gelokaliseerd in de bovenste luchtwegen, vagina en mogelijk ook in de huid, betrokken zijn bij deze exocrinopathie. De klinische verschijnselen van SS vloeien voort uit de onderliggende exocrinopathie en omvatten o.a. typische sicca symptomen als droge ogen en een droge mond. Daarnaast wordt SS klinisch gekarakteriseerd door niet-specifieke “griep-achtige” verschijnselen zoals koorts, gewrichtspijn, spierpijn en vermoeidheid. Er wordt geschat dat SS aanwezig is in circa 0.5-1.0% van de bevolking in de USA en Europa, en het ziektebeeld is reeds onderwerp geweest van uitgebreid wetenschappelijk onderzoek. Eén van de continu aanwezige dilemma's zowel bij onderzoek als in de dagelijkse klinische praktijk, is hoe het SS gedefinieerd moet worden. Dientengevolge kunnen resultaten verkregen bij onderzoek niet eenvoudig met elkaar vergeleken worden. Van belang is tevens dat in de dagelijkse klinische praktijk SS door sommige artsen gediagnosticeerd wordt aan de hand van classificatie criteria, terwijl andere artsen er de voorkeur aan geven SS te diagnosticeren op basis van klinische verschijnselen die onderdeel vormen van het syndroom dat oorspronkelijk beschreven is door Henrik Sjögren, een Zweedse oogarts.

Een algemene inleiding op dit proefschrift wordt in **Hoofdstuk 1** gegeven. In dit hoofdstuk wordt een historisch overzicht gegeven van het zich in de tijd ontwikkelende concept van SS, startend met de eerste beschrijvingen van het syndroom door Henrik Sjögren en anderen, gevolgd door een beschrijving van diverse voorgestelde classificatie criteria en opeenvolgende modificaties hier aan. De problemen die op dit moment ondervonden worden bij het ontwikkelen van een objectief model voor de diagnose van SS en het bepalen van de ziekte-activiteit in SS worden tevens in dit hoofdstuk besproken en vormen de achtergrond voor dit proefschrift.

In de huidige 2002 *American/European Consensus Group* (AECG) classificatie criteria voor SS worden objectieve items, zoals de aanwezigheid van anti-SS-A of anti-SS-B antistoffen (anti-SS-A, anti-SS-B) in het serum, benadrukt. In het serum van circa 20% van de SS patiënten wordt echter geen anti-SS-A of anti-SS-B gedetecteerd op het moment van het stellen van de diagnose. In deze ‘seronegatieve’ patiënten kan anti-SS-A of anti-SS-B niettemin elders in het lichaam aanwezig zijn, bv. in de exocriene klieren zelf of in hun excreta als traanvocht of speeksel. In **Hoofdstuk 2** wordt een studie beschreven welke gericht is op deze relatief weinig bestudeerde groep van anti-SS-A en anti-SS-B seronegatieve SS patiënten. Het doel van deze studie was om vast te stellen of anti-SS-A en anti-SS-B gedetecteerd zouden kunnen worden in traanvochtmonsters van onze seronegatieve SS patiënten populatie, en om na te gaan of seroconversie naar een positief serologisch profiel optreedt als de ziekte minimaal vijf jaar voortgeschreden is sinds het stellen van de diagnose. Als één van beiden het geval blijkt te zijn dan zou de “intrinsieke” sensitiviteit van anti-SS-A en anti-SS-B hoger kunnen zijn dan tot nu toe is aangenomen. De resultaten van deze studie tonen dat er geen aanwijzingen zijn dat seroconversie van een negatief naar

positief anti-SS-A en anti-SS-B serologisch profiel plaatsvindt in het beloop van SS. Wel is gebleken dat in sommige seronegatieve SS patiënten anti-SS-A aanwezigheid wel kan worden aangetroffen in traanvochtmonsters. In seropositieve SS patiënten die als controle dienden werd zowel anti-SS-A als anti-SS-B aanwezigheid aangetoond in traanvochtmonsters. Een opmerkelijk klein volume aan traanvocht (circa 5 µl) bleek reeds voldoende te zijn om deze antistoffen te kunnen aantonen met behulp van ELISA. Daarbij dient wel aangetekend te worden dat het aantal onderzochte seronegatieve patiënten klein was en de gemeten sensitiviteit eveneens laag was. De toegevoegde diagnostische waarde van traanvocht analyse op aanwezigheid van anti-SS-A of anti-SS-B in seronegatieve SS patiënten dient derhalve nog bewezen te worden.

Implicaties van deze studie voor de klinische praktijk zijn:

- Herhaald serologisch onderzoek op de aanwezigheid van anti-SS-A of anti-SS-B in voorheen seronegatieve patiënten heeft geen toegevoegde diagnostische waarde.
- Hoewel geoptimaliseerde traanvocht analyse anti-SS-A of anti-SS-B aanwezigheid kan onthullen in seronegatieve SS patiënten, daarmee het diagnosticeren van SS faciliterend, is de opbrengst van traanvocht analyse in deze patiënten thans te laag om aan te bevelen in de klinische praktijk

De eerder gerapporteerde ziekte-sensitiviteit van anti-SS-A en anti-SS-B bedraagt respectievelijk 60-75 % en 30-50 %, terwijl de ziekte-specificiteit van met name anti-SS-B algemeen als zijnde tamelijk hoog beschouwd wordt. Anti-SS-B wordt voornamelijk aangetroffen in SS en SLE patiënten, en is zelden aanwezig in andere ziekten of in de normale gezonde populatie. Niettemin, zoals hierboven reeds gemeld, ontbreekt in circa 20% van de SS patiënten serum aanwezigheid van anti-SS-A en anti-SS-B. In verband met deze beperkte sensitiviteit en specificiteit, wordt de zoektocht naar antistoffen met een beter sensitiviteit- en specificiteit profiel voortgezet. Anti-alpha-fodrine autoantistoffen (anti-alpha-fodrine) als serologische *marker* voor SS vormen één van de voorgestelde kandidaten. Van het sensitiviteit- en specificiteit profiel van anti-alpha-fodrine is eerder geclaimd dat het superieur zou zijn aan dat van anti-SS-A en anti-SS-B. In de oorspronkelijke studies werd een sensitiviteit van 96% gerapporteerd van anti-alpha-fodrine in het primaire SS, terwijl de specificiteit vergelijkbaar zou zijn met die van anti-SS-B. De resultaten gepresenteerd in **Hoofdstuk 3** laten echter zien dat de voorheen gerapporteerde sensitiviteit en specificiteit van anti-alpha-fodrine niet gereproduceerd konden worden in ons cohort van goed gedefinieerde SS patiënten. Deze conclusie is gebaseerd op meerdere ELISA metingen welke met minimal één alternatieve biochemische meettechniek bevestigd werden. In tegenstelling tot anti-alpha-fodrine, konden alle anti-SS-A en anti-SS-B positieve sera in de ELISA bevestigd worden d.m.v. alternatieve biochemische technieken. Voorts was de geobserveerde sensitiviteit en specificiteit van anti-SS-A en anti-SS-

B hoger dan die van anti-alpha-fodrine. Bovendien bleek een aanzienlijke overlap te bestaan tussen aanwezigheid van anti-SS-A, anti-SS-B en anti-alpha-fodrine antistoffen. Een potentieel bijdragende rol van anti-alpha-fodrine bepaling om SS patiënten te detecteren die anti-SS-A en anti-SS-B seronegatief zijn, lijkt daarom onwaarschijnlijk. Hoewel het studiemodel niet gericht was op het vaststellen van ziekte specificiteit lijkt het voorts onwaarschijnlijk dat de specificiteit van anti-alpha-fodrine die van anti-SS-B overstijgt, gezien de detectie van enkele anti-alpha-fodrine positieve sera in reumatoïde artritis (RA) patiënten.

Het bepalen van anti-alpha-fodrine antistoffen voegt derhalve niet veel toe aan de diagnose van het syndroom van Sjögren.

In het kader van het huidige concept van SS als auto-immuun exocrinopathie van onbekende origine, wordt verondersteld dat frequent gerapporteerde xerosis (droge huid) het gevolg is van cutane exocrinopathie. Overtuigend histologisch bewijs voor deze hypothese ontbreekt echter. Theoretisch gezien zou een huidbiopt als diagnosticum kunnen dienen als een alternatief voor het lipbiopt mits de typische infiltrerende lymfocyten aggregaten, die gezien worden in speekselklierbiopten ook in de huid worden aangetroffen.

Exocriene klieren in de huid omvatten talgklieren en eccriene alsmede merocriene (voorheen apocrien genoemde) zweetklieren. Talg is, veel meer dan zweet, betrokken bij het reguleren van vochtverlies uit de epidermis. Een afgenomen talgklier functie weerspiegelt zich dan ook in xerosis. Interessant is dat alle exocriene klieren in de menselijke huid (mede) gereguleerd worden door geslachtshormonen. Hoewel SS voornamelijk bij vrouwen voorkomt is er tot dusverre nimmer een direct door oestrogenen gemedieerd onderliggend pathofysiologisch mechanisme geïdentificeerd. In **Hoofdstuk 4** worden huidbiopten van SS patiënten onderzocht op tekenen van exocrinopathie. Tevens worden in dit hoofdstuk de oestrogeen-alpha ( $ER\alpha$ ) en oestrogeen-beta ( $ER\beta$ ) receptor expressie in huid- en speekselklierbiopten van SS patiënten, in vergelijking met controle biopten van gezonde vrijwilligers, beschreven. Er werd geen bewijs gevonden voor het bestaan van inflammatoire cutane exocrinopathie in SS.

In de klinische praktijk is derhalve geen rol weggelegd voor een huidbiopt als alternatief voor het lipbiopt bij het diagnosticeren van het syndroom van Sjögren.

In SS patiënten blijkt dat  $ER\beta$  de overheersend aanwezige ER in huid en speekselklier weefsel is. Dit komt overeen met de eerder beschreven bevindingen in huid en speekselklier weefsel van gezonde vrijwilligers. Het in dit onderzoek geobserveerde gedifferentieerde  $ER\beta$  expressie patron en de aanwezigheid van  $ER\beta$  positieve lymfocyten in de speekselklier infiltraten in SS patiënten zou op een rol voor  $ER\beta$  in de pathogenese of het onderhouden van SS kunnen wijzen.

Aanvullende studies moeten echter uitwijzen of deze observatie van pathofysiologische betekenis is. Indien dit het geval is kunnen nieuwe pathofysiologische en therapeutische concepten voor het SS ontwikkeld worden

Naast de aanwezigheid van anti-SS-A of anti-SS-B in sera van patiënten vormt ook een positief speekselklierbiopt een objectief item waaraan extra gewicht is toegekend in de AECG criteria. Een speekselklierbiopt wordt thans beschouwd als positief indien de lymfocyttaire focus score (LFS) groter dan, of gelijk is aan 1. De LFS representeert het aantal mononucleaire cel infiltraten waarin minimaal 50 inflammatoire cellen per 4 mm<sup>2</sup> aanwezig zijn. De beperkte ziekte sensitiviteit en specificiteit van de LFS is echter alom bekend. Additioneel onderzoek van het speekselklierbiopt kan daarom helpen te differentiëren tussen ware en valse positieve of negatieve LFS waarden. Eén van de methoden hiervoor is kwantitatief immunohistologisch (KIH) onderzoek van het speekselklierbiopt. Hierbij wordt de samenstelling van het infiltraat beschreven naar percentage van immunoglobuline subtypes (IgG, IgM, en IgA) bevattende cellen. In het KIH criterium, geïntroduceerd in 1982 door Bodeutsch et al. wordt een percentage IgA bevattende plasma cellen (IgA%) kleiner dan 70 als zeer specifiek voor SS beschouwd. Met behulp van het KIH criterium konden enkele patiënten die voorheen niet voldeden aan de classificatie criteria i.v.m. een LFS < 1 alsnog geïdentificeerd worden als SS patiënten. Daarnaast zijn eerder patiënten beschreven met een auto-immuun ziekte zoals RA en een LFS>1 (sialadenitis) maar zonder klinische verschijnselen van SS, en deze patiënten voldoen vaak niet aan het KIH criterium. Het lijkt dus aanbevelenswaardig om speekselklierbiopten immunohistologisch te analyseren in plaats van, of gecombineerd met, histologische evaluatie.

In **Hoofdstuk 5** wordt de ziekte-sensitiviteit en –specificiteit van histologische (LFS) versus immunohistologische (IgA%) resultaten van speekselklierbiopten geëvalueerd. In een ruime meerderheid van de gevallen blijkt zowel de LFS als het IgA% tot dezelfde ondubbelzinnige histologische conclusie te leiden. Niettemin, of beide parameters nu overeenkomen of niet, geen van beiden heeft een sensitiviteit of specificiteit van 100%, zoals ook naar voren komt uit een analyse van concordante biopten. In 11% van de gevallen stemde de conclusie van histologisch onderzoek (LFS) niet overeen met die van immunohistologisch onderzoek (IgA%). In deze zogenoemde “mismatch” gevallen lijkt het IgA% superieur aan de LFS wanneer objectieve, serologische bevindingen in ogenschouw genomen worden. Differentiatie tussen sialadenitis secundair aan RA versus Sjögren's syndrome werd bevorderd door meer waarde toe te kennen aan een verhoogd gammaglobuline en de aanwezigheid van anti-SS-A en anti-SS-B. Deze objectieve parameters lijken meer SS-specifiek te zijn dan de reumafactor en ANA, welke ook frequent aanwezig zijn in bv. RA patiënten.

Deze resultaten suggereren dat een accurate diagnose van het syndroom van Sjögren in de klinische praktijk bevorderd kan worden door zowel LFS als IgA% van het speekselklierbiopt in ogenschouw te nemen. In twijfelgevallen kan dan de tweede parameter naar de juiste diagnose wijzen. In dit onderzoek was het aantal vals-negatieve IgA% scores opmerkelijk laag. Een IgA% >70 gecombineerd met een LFS >1 zou daarom uitgelegd moeten worden als een vals-positieve LFS. De interpretatie van een LFS <1 in gecombineerd met een IgA% <70 is minder helder, aangezien zowel vals-negatieve LFS als vals-positieve IgA% scores ongeveer even frequent voorkomen.

Hoewel er momenteel geen effectieve systemische behandeling voor SS beschikbaar is, moet aangetekend worden dat in klinische trials het therapeutisch effect vooral klinisch geëvalueerd wordt, waarbij gebruik gemaakt wordt van een scala aan niet-uniforme objectieve en subjectieve parameters. De behoefte aan een objectieve maat voor het meten van ziekte-activiteit is dan ook groot.

*Reversibiliteit* is een belangrijke component van het momenteel voorgestelde ziekte-activiteit model. Goed gedocumenteerde kinische of histologische reversibiliteit is echter nog niet gerapporteerd in SS onderzoek.

In **Hoofdstuk 6** worden de resultaten beschreven van (immuno)histologisch onderzoek van een speekselklierbiopt van een SS patiënt voor en na behandeling met een hoge dosering corticosteroiden. Zowel LFS als IgA% waren suggestief voor SS voor starten van de behandeling, en normaliseerden na behandeling. Deze casus suggereert dat (immuno)histologische veranderingen in SS reversibel kunnen zijn. Een herhaald speekselklierbiopt zou dus een objectief instrument kunnen zijn voor het evalueren van het therapie effect in klinische trials.

In de klinische praktijk moet er rekening mee gehouden worden dat vals-negatieve (immuno)histologische speekselklierbiopt uitslagen kunnen optreden bij gelijktijdige behandeling met corticosteroiden.

Om de potentiële reversibiliteit van immunohistologische veranderingen in speekselklierbiopten nader te onderzoeken, en de klinische therapeutische effectiviteit te evalueren, werd een pilot study uitgevoerd waarin SS patiënten werden behandeld met etanercept (Enbrel®) (**Hoofdstuk 7**). Etanercept (Enbrel®), een oplosbare volledig humane TNF- $\alpha$ -p75-receptor fusie proteïne, interfereert met het ontstekingsproces door het pro-inflammatoire cytokine TNF- $\alpha$  te binden en te inactiveren. Omdat toegenomen TNF- $\alpha$  expressie geobserveerd is in exocriene klieren van SS patiënten, zou anti-TNF- $\alpha$  behandeling in SS patiënten potentieel het ontstekingsproces kunnen onderdrukken met lokale en systemische effecten. Uit de resultaten van deze pilot study moest echter geconcludeerd worden dat etanercept, subcutaan toegediend in een conventionele RA dosis van 25 mg tweemaal per week, niet heeft geleid tot een afname van sicca klachten en verschijnselen in de 15 deelnemende SS patiënten. Evenmin resulteerde dit etanercept doseringsschema in



statistisch significante histologische veranderingen in speekselklierbipten. In tegenstelling tot behandeling met corticosteroïden zijn er dus geen aanwijzingen dat het resultaat van een diagnostisch speekselklierbipt verstoord wordt door gelijktijdige behandeling met etanercept.

In de klinische praktijk biedt etanercept in een doseringsschema van 25 mg tweemaal per week geen effectieve behandelingsvorm voor het syndroom van Sjögren. Vanuit diagnostisch oogpunt lijkt gelijktijdige etanercept behandeling niet van invloed te zijn op de resultaten van het speekselklierbipt.

### **Conclusie en afsluitende opmerkingen**

De verschillende onderdelen van dit proefschrift kunnen allen gezien worden in de context van het zich ontwikkelende SS concept, dat thans met name gericht is op het verbeteren van de bestaande classificatie criteria en de ontwikkeling van een objectieve meetinstrument voor ziekte-activiteit. In de meest recente wijziging van de SS classificatie criteria, de 2002 AECG criteria, wordt extra gewicht toegekend aan objectieve verschijnselen zoals aanwezigheid van anti-SS-A of anti-SS-B en een positief speekselklierbipt. Voor beide objectieve parameters geldt echter dat ze gelimiteerd zijn in ziekte-specificiteit en ziekte-sensitiviteit. Het verder ontwikkelen van classificatie criteria, welke een hogere mate van ziekte-specificiteit en ziekte-sensitiviteit bevatten blijft daarom nodig.

In dit proefschrift werden zowel de serologische als histologische items in de huidige classificatie criteria nader bestudeerd. Van anti-alpha fodrine antistoffen kon niet worden aangetoond dat ze superieur waren aan anti-SS-A en anti-SS-B. Anti-alpha fodrine antistoffen zouden de in de huidige classificatie criteria aanwezige anti-SS-A en anti-SS-B dan ook niet moeten vervangen. De ziekte-sensitiviteit en ziekte-specificiteit van anti-SS-A en anti-SS-B blijft echter ook gelimiteerd. In circa 20 procent van de SS patiënten zijn deze antistoffen afwezig in het serum, en dat blijft ook zo naarmate de ziekte voortschrijdt. Hoewel anti-SS-A en anti-SS-B ook gedetecteerd kunnen worden in speeksel en traanvocht van SS patiënten is het nog maar de vraag of analyse van deze excreta van exocriene klieren daadwerkelijk zal leiden tot een hogere ziekte-sensitiviteit van deze antistoffen.

Een positief speekselklierbipt is thans gedefinieerd als een minimum LFS van 1. Voorheen werd echter reeds in meerdere studies aangetoond dat zowel de ziekte-specificiteit als de ziekte-sensitiviteit van de LFS als histologische maat matig is. Een manier om deze te verbeteren kan gevonden worden door de compositie van het speekselklier infiltraat nader te evalueren, bv. door het bepalen van het percentage IgA bevattende plasmacellen. Er werd aangetoond dat het gecombineerd bepalen van IgA% en LFS tot een meer accurate diagnose van het SS kan leiden.

Een ziekte-activiteit model voor SS is thans in ontwikkeling. *Reversibiliteit* is een belangrijke karakteristiek in het voorgestelde model. Reversibiliteit is echter nog niet vaak op objectieve wijze gedocumenteerd in SS. Het milde, langzaam progressieve

karakter van het ziektebeloop in ogenschouw nemend, alsmede de lage frequentie van door patiënten zelf gerapporteerde *flares* (opvlammingen van het ziektebeeld), doet de vraag rijzen of ziekte-activiteit wel daadwerkelijk meetbaar is in SS. Mogelijk is een en ander een gevolg van het feit dat wanneer SS klinisch manifest wordt en de diagnose gesteld wordt patiënten zich reeds in een eindstadium van het ziektebeeld bevinden met weinig uitzicht op reversibiliteit van reeds aanwezige exocrinopathie. Het lijkt wellicht meer voor de hand te liggen te starten met het definiëren van een schade-index waarin de ernst van de ziekte (*disease severity* of *damage index*) tot uiting wordt gebracht, gegeven het stabiele klinische beloop in SS patiënten. Tot nu toe is er echter m.b.t. eind-orgaan schade in SS slechts een enkele studie gerapporteerd.

Teneinde SS te diagnosticeren voordat irreversibele schade aan exocriene klieren ontstaat, zou een screenende test voor SS ontwikkeld moeten worden welke toegepast kan worden in een “pre-sicca” stadium waarin de patiënt zich nog slechts met niet-specifieke symptomen presenteert. Dit vraagt om een snelle, goedkope en bij voorkeur niet-invasieve testmethode om SS uit te sluiten danwel aan te tonen in potentieel grote patiënten populaties. Echter, de behoefte aan vroegdiagnostiek (voordat irreversibele schade heeft plaatsgevonden) wordt mogelijk niet breed ervaren totdat een curatieve behandeling, welke het mogelijk maakt te interveniëren in het beloop van het syndroom van Sjögren, beschikbaar komt. Tot die tijd lijkt vroegdiagnostiek, bij gebrek aan bewezen curatieve therapie, weinig consequenties te hebben.

## APPENDIX

### **Sjogren's syndrome criteria [letter]**

Ann Rheum Dis 2002;61:948-949

In ARD 6/2002 Manthorpe comments on the recently proposed US-European classification criteria for Sjögren's syndrome (SS)<sup>1</sup>. We would like to address some of the issues he raises, and add some comments.

Now that the classification criteria have evolved from rather subjectively biased ones to more objective assessments, it is surprising that the two most disease-specific objective parameters currently available for SS are subject to considerable criticism. Of course when serological and histological items are emphasised in the new SS classification criteria, their individual disease sensitivity and specificity should always be kept in mind.

In fact all 6 items that are included in the classification criteria can be subject to discussion. E.g. the Schirmer-1 test, and unstimulated whole salivary flow test have also been criticised in a number of papers<sup>2-5</sup>, but these items are rather recommended in Manthorpe's paper.

Regarding sublabial salivary gland biopsies (SLGBs) Manthorpe expresses his concerns about their accuracy referring to one paper in which over 50% change of diagnosis after second examination of SLGBs is reported. However, the authors themselves report that not using the focus scoring system was probably the most important reason for the change of diagnosis on second examination. They did not conclude that the focus score itself –which is mandatory to fulfil item VI- changed dramatically upon re-examination of the specimens !

Other ways of bypassing inter-observer variability are also available e.g. measuring two instead of one parameter (e.g. IgA% and focus score) has a synergistic value for the accuracy of diagnosis<sup>6</sup>, and moreover computer-aided scoring methods may provide non-observer dependent data. For measuring the IgA% reliable and reproducible objective data from the biopsies are obtained by combining microscope, computer and calibrated software. These biopsies show what is going on in the target organs of this disease and may provide early diagnostic markers; one should not put them aside too easily. Manthorpe also criticises the SS classification criteria for the interdependent relation between anti-SS-A/anti-SS-B antibodies (item IV) and the focus score (item VI). They are certainly associated to each other but why is that a problem ? The world-wide accepted ARA criteria for rheumatoid arthritis contain interdependent items as well. E.g. positive rheumatoid factor serology is generally considered as strongly associated with radiological joint damage. Interdependency can also be found in the ACR classification criteria for SLE (presence of ANA is a distinct item from presence of anti-dsDNA or anti-Sm, items 11 and 10 respectively). Furthermore it appears inconsistent that Manthorpe recommends including the patient's smoking habits in the SS classification criteria.

This would introduce an interdependent item as well. Also the dependency does not equal a one-on-one relation, i.e. seronegative patients may have a positive focus score and vice versa. Especially since numerous reports have shown that the focus score alone can be false-positive or false-negative<sup>7-10</sup>, the presence of anti-SS-A/anti-SS-B antibodies, which are still the most disease specific and sensitive parameters available, has additional value for the accuracy of diagnosis. Finally, it has yet to be proven that the suggested new antibodies (anti-fodrin, anti-muscarin) are more sensitive and disease specific than the existing classic anti-SS-A and anti-SS-B antibodies. Therefore it is too early to include such items in classification criteria. Whilst our knowledge of Sjögren's syndrome increases, classification criteria may develop in a way that enhances early diagnosis of possibly reversible target organ damage. Therefore not the end-stage symptoms and signs (items I to III and V) but rather the early target organ histological signs and serological signs are likely to retain their place in the classification criteria. Therefore, in our view the US-European consensus group is right to emphasise item IV and VI which should not be neglected until superior alternatives have been introduced.

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Reumatoloog J. Wentink had voorheen tijdens haar eigen onderzoeksperiode waardevol werk verricht voor mij, omdat ik direct gebruik kon maken van een uitgebreide database met gegevens van Sjögren syndroom patiënten in het UMCN.

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## Curriculum vitae

Michiel Maarten Zandbelt is geboren op 25 november 1971 te Doesburg waar hij opgroeide en zijn ouders nog immer woonachtig zijn. Voor de middelbare school fietste hij 6 jaar lang 12 km naar Doetinchem (v.v.). Aldaar behaalde hij in 1990 het V.W.O examen aan het het Ludgercollege. In 1991 behaalde hij het propaedeutisch examen Psychologie aan de Radboud Universiteit te Nijmegen. In datzelfde jaar werd hij alsnog ingeloot voor de studie Geneeskunde welke hij, eveneens in Nijmegen, succesvol afrondde met het artsexamen in 1998. Na een keuze co-assistentenschap reumatologie en een wetenschappelijke stage binnen dit vakgebied, was hij van 1998-2002 werkzaam als arts-onderzoeker reumatologie in het Universitair Medisch Centrum Nijmegen onder begeleiding van prof. P. van Riel. Behalve een nadere kennismaking met reumatische patiënten in klinische trials en internationaal wetenschappelijk congresbezoek, bood deze periode hem de gelegenheid samen met dr. F. van den Hoogen en prof. L. van de Putte de basis te leggen voor het onderzoek dat heeft geleid tot dit proefschrift. Van 2002-2005 was hij in het kader van de specialisatie tot reumatoloog werkzaam als arts-assistent interne geneeskunde in het Jeroen Bosch Ziekenhuis te 's Hertogenbosch onder leiding van internist dr. P. Netten. Van februari 2006 tot mei 2007 was hij werkzaam als reumatoloog-in-opleiding in de Sint Maartenskliniek te Nijmegen onder supervisie van dr. M. Franssen. Voor het vervolg van de specialisatie tot reumatoloog is hij per 1 mei 2007 werkzaam in ziekenhuis Rijnstate te Arnhem onder supervisie van dr. M. Janssen.

Michiel is getrouwd met Helen de Vries, psychologe, en samen zijn zij sinds oktober 2005 de trotse ouders van hun tweelingdochters Emma en Joy.

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